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(71) Applicants: GENZYME CORPORATION [US/US]; One Mountain Road, Framingham, MA 01701 (US). UNIVERSITY OF IOWA RESEARCH FOUNDATION [US/US]; Oakdale Research Campus #214 TIC, Iowa City, IA 52242-5000 (US).

(72) Inventors: FASBENDER, Allen, J.; 2765 Lakeview Drive N.E., Solon, IA 52333 (US). WELSH, Michael, J.; 3460 560th Street, Riverside, IA 52327 (US). SIEGEL, Craig, S.; 15 Bradford Road, Woburn, MA 01801 (US). LEE, Edward, R.; 175-J Center Street, Lincoln Heights, Quincy, MA 02169 (US). CHANG, Chau-Dung; 7 Middleby Road, Lexington, MA 02169 (US). MARSHALL, John; 45 West Walnut Street, Milford, MA 01757 (US). CHENG, Seng, H.; 50 Wall Street, Wellesley, MA 02181 (US). HARRIS, David, J.; Eastern Avenue, Lexington, MA 02173 (US). EASTMAN, Simon, J.; 13 Royal Crest Drive; Marlboro, MA 01752 (US). HUBBARD, Shirley, C.; 26 Wiley Road, Belmont, MA 02178 (US). LANE, Mathieu, B.; 55 Sacramento Street, Cambridge, MA 02138 (US). ROWE, Eric, A.; 196 Sylvan Street, Malden, MA 02148 (US). SCHEULE, Ronald, K.; 26 East Street, Hopkinton, MA 01748 (US). YEW, Nelson, S.; 25 Rockdale Hill Circle, West Upton, MA 01568 (US).

(74) Agent: LASSEN, Elizabeth; Genzyme Corporation, One Mountain Road, Framingham, MA 01701 (US).

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(57) Abstract

Novel compositions are provided. Typically, the compositions comprise one or more neutral co-lipids and also a cationic amphiphile. Therapeutic compositions are prepared according to the practice of the invention by contacting a therapeutically active molecule with a dispersion of neutral co-lipid(s) and amphiphile(s).

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Compositions Comprising Cationic Amphiphiles and Co-lipids for Intracellular Delivery of Therapeutic Molecules

Background of the Invention

The present invention relates to novel lipid compositions that facilitate the intracellular delivery of biologically active (therapeutic) molecules. The present invention relates also to pharmaceutical compositions that comprise such lipid compositions , and that are useful to deliver therapeutically effective amounts of biologically active molecules into the cells of patients.

Effective therapeutic use of many types of biologically active molecules has not been achieved simply because methods are not available to cause delivery of therapeutically effective amounts of such substances into the particular cells of a patient for which treatment therewith would provide therapeutic benefit. Efficient delivery of therapeutically sufficient amounts of such molecules into cells has often proved difficult, if not impossible, since, for example, the cell membrane presents a selectively-permeable barrier. Additionally, even when biologically active molecules successfully enter targeted cells, they may be degraded directly in the cell cytoplasm or even transported to structures in the the cell, such as lysosomal compartments, specialized for degradative processes. Thus both the nature of substances that are allowed to enter cells, and the amounts thereof that ultimately arrive at targeted locations within cells, at which they can provide therapeutic benefit, are strictly limited.

Although such selectivity is generally necessary in order that proper cell function can be maintained, it comes with the disadvantage that many therapeutically valuable substances (or therapeutically effective amounts thereof) are excluded. Additionally, the complex structure, behavior, and environment presented by an intact tissue that is targeted for intracellular delivery of biologically active molecules often interfere with such delivery, in comparison with the case presented by populations of cells cultured in vitro .

Examples of biologically active molecules for which effective targeting to a patients' tissues is often not achieved include: (1) numerous proteins including immunoglobin proteins, (2) polynucleotides such as genomic DNA, cDNA, or mRNA (3) antisense polynucleotides; and (4) many low molecular weight compounds, whether synthetic or naturally occurring, such as the peptide hormones and antibiotics.

One of the fundamental challenges now facing medical practicioners is that although the defective genes that are associated with numerous inherited diseases (or that represent disease risk factors, including for various cancers) have been isolated and characterized, methods to correct the disease states themselves by providing patients with normal copies of such genes (the techniques of gene therapy) are substantially lacking. Accordingly, the development of improved methods of intracellular delivery is of great medical importance.

Examples of diseases that it is hoped can be treated by gene therapy include inherited disorders such as cystic fibrosis, Gaucher's disease, Fabry's disease, and muscular dystrophy. Representative of acquired disorders that can be treated are: (1) for cancers — multiple myeloma, leukemias, melanomas, ovarian carcinoma and small cell lung cancer; (2) for cardiovascular conditions — progressive heart failure, restenosis, and hemophilias; and (3) for neurological conditions — traumatic brain injury.

Gene therapy requires successful transfection of target cells in a patient. Transfection may generally be defined as the process of introducing an expressible polynucleotide (for example a gene, a cDNA, or an mRNA patterned thereon) into a cell. Successful expression of the encoding polynucleotide leads to production in the cells of a normal protein and leads to correction of the disease state associated with the abnormal gene. Therapies based on providing such proteins directly to target cells (protein replacement therapy) are often ineffective for the reasons mentioned above.

Cystic fibrosis, a common lethal genetic disorder, is a particular example of a disease that is a target for gene therapy. The disease is caused by the presence of one or more mutations in the gene that encodes a protein known as cystic fibrosis transmembrane conductance regulator ("CFTR"), and which regulates the movement of ions (and therefore fluid) across the cell membrane of epithelial cells, including lung epithelial cells. Abnormnal ion transport in airway cells leads to abnormal mucous secretion, inflammmation and infection, tisssue damage, and eventually death.

It is widely hoped that gene therapy will provide a long lasting and predictable form of therapy for certain disease states, and it is likely the only form of therapy suitable for many inhereted diseases. There remains however a critical need to provide new lipid compositions that further faciliate entry of functional genes into cells.

Reported Developments

In as much as compounds designed to facilitate intracellular delivery of biologically active molecules must interact with both non-polar and polar environments (in or on, for example, the plasma membrane, tissue fluids, compartments within the cell, and the biologically active molecule itself), such compounds are designed typically to contain both polar and non-polar domains. Compounds having both such domains may be termed

amphiphiles, and many lipids and synthetic lipids that have been disclosed for use in facilitating such intracellular delivery (whether for in vitro or in vivo application) meet this definition. One particularly important class of such amphiphiles is the cationic amphiphiles. In general, cationic amphiphiles have polar groups that are capable of being positively charged at or around physiological pH, and this property is understood in the art to be important in defining how the amphiphiles interact with the many types of biologically active (therapeutic) molecules including, for example, negatively charged polynucleotides such as DNA.

Examples of cationic amphiphilic compounds that have both polar and non-polar domains and that are stated to be useful in relation to intracellular delivery of biologically active molecules are found, for example, in the following references, which contain also useful discussion of (1) the properties of such compounds that are understood in the art as making them suitable for such applications, and (2) the nature of structures, as understood in the art, that are formed by complexing of such amphiphiles (lipids) with therapeutic molecules intended for intracellular delivery.

(1) Felgner, et al., Proc. Natl. Acad. Sci. USA, 84, 7413-7417 (1987) disclose use of positively-charged synthetic cationic lipids including N-[1(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride ("DOTMA"), to form lipid/DNA complexes suitable for transfections. See also Felgner et al., The Journal of Biological Chemistry, 269(4), 2550-2561 (1994). Generally, such compounds are characterized as having a 2,3-dihydroxypropylamine backbone whereby the amine (and modifications thereto) provide the cationic portion and long chain alkyl groups attached to the hydroxyl oxygen atoms provide the lipid moiety.

(2) Behr et al., Proc. Natl. Acad. Sci. USA, 86, 6982-6986 (1989) disclose numerous amphiphiles characterized by a carboxypermine moiety attached to

lipidic groups via an amidoglycyl linker , including dioctadecyl-amidologlycylspermine ("DOGS").

(3) U.S. Patent 5, 283,185 to Epand et al. describes additional classes and species of amphiphiles including 3ß [N-(N^1,N^1 - dimethylaminoethane)-carbamoyl] cholesterol, termed "DC-chol". Such amphiphiles are characterized generally, by use of cholesterol as the lipophilic group, which may then be attached to an alkylamine moiety through one of several linkers, including carboxyamide and carbamoyl.

(4) Additional compounds that facilitate transport of biologically active molecules into cells are disclosed in U.S. Patent No. 5,264,618 to Felgner et al. See also Felgner et al., The Journal Of Biological Chemistry, 269(4), pp. 2550-2561 (1994) for disclosure therein of further compounds including "DMRIE" 1,2- dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide, which is discussed below.

(5) Additional reference to amphiphiles suitable for intracellular delivery of biologically active molecules is also found in U.S. Patent No. 5,334,761 to Gebeyehu et al., and in Felgner et al., Methods(Methods in Enzymology), 5, 67- 75 (1993).

Although the compounds mentioned in the above-identified references have been demonstrated to facilitate (although in many such cases only *in vitro*) the entry of biologically active molecules into cells, it is believed that the uptake efficiencies provided thereby are insufficient to support numerous therapeutic applications, particulary gene therapy. Additionally, since the above-identified compounds are understood to have only modest activity, substantial quantities thereof must be used-leading to concerns about the toxicity of such compounds or of the metabolites thereof. Accordingly there is a need to develop further generations of cationic amphiphiles whose activity is sufficient that successful therapies can be achieved therewith.

It is generally recognized that the activity of cationic amphiphiles can be improved if they are provided as mixtures with one or more neutral co-lipids. For the purposes of the invention, a neutral co-lipid is defined as a phosopholipid capable of existing as a zwitterion at physiological pH. A neutral co-lipid that is well recognized in the art for this purpose is dioleoyl-sn-glycero-3-phosphoethanolamine ("DOPE").

Although it is recognized that DOPE is effective with many cationic amphiphiles to facilitate entry of therapeutcally active molecules into cells, given the requirement that uptake efficiencies (and, for example, in the case of an encoding DNA, subseqeunt expression) of the therapeutic molecule must be high to support therapeutic applications, it is desireable to identify new amphiphile /co-lipid compositions that are even more effective in this capacity.

Summary of the Invention

It is generally recognized that the capability of cationic camphiphiles to deliver biologically(therapeutically) active molecules into cells may be further enhanced by providing amphiphile compositions that also contain neutral co-lipids. Without being limited as to theory, it is believed that particular neutral co-lipids have, for example, certain properties (including net charge, shape or effective size) that compliment those of cationic amphiphiles, and thus act to alter the manner in which cationic amphiphiles will assemble to form, for example, micelles, bilayers, liposomes, and hexagonal array structures. Such properties affect how a complex (for example, of DNA and cationic amphiphile) will behave in cells, including how the composition may enter a cell, or escape from any compartment within the cell (such as an endosomal compartment) where it may be subject to processing. Accordingly, the present invention provides for novel lipid compositions that contain one or more cationic amphiphiles and one or more neutral co-lipids.

Generally speaking, the performance characteristics of most cationic amphiphiles can be improved by providing them for therapeutic use with the co-lipids described below. It is believed also that many such therapeutic compositions provide performance characteristics that are substantially better that those achieved when dioleoyl-sn-glycero-3-phosphoethanolamine ("DOPE") is used as co-lipid. In fact, for certain amphiphiles, reporter gene expression achieved in vivo has been improved 5 to 10 fold using the amphiphile/neutral co-lipid compositions of the invention, compared to that measured when DOPE was selected as co-lipid. The teachings of the present invention are of particular value with respect to use of cationic amphiphiles containing steroid lipophilic groups, and relatively large (hydrated) polar domains.

Accordingly there are provided pharmaceutical compositions that comprise (1) therapeutically active molecules (such as a ribosomal RNA; an antisense polynucleotide whether of RNA or DNA; a ribozyme; and a polynucleotide of genomic DNA, cDNA, or mRNA that encodes for a therapeutically useful protein); and (2) one or more cationic amphiphiles and particular neutral co-lipids, that facilitate entry of the therapeutically active molecules into cells.

Representative of the neutral co-lipids useful in the practice of the invention are:

(1) a 1-acyl-2-acyl-sn-glycero-3-phosphoethanolamine having at least two positions of unsaturation in each acyl (fatty acid) chain thereof wherein said acyl chains have, individually, from about 12 to about 22 carbon atoms, and are the same (symmetric) or different (asymmetric), and wherein each of the double bonds in the acyl chains of the 1-acyl-2-acyl-sn-glycero-3-phosphoethanolamine is, independently, either cis or trans; with examples thereof including neutral co-lipids in which the 1-acyl and 2-acyl chains, respectively, are independently selected from the group consisting of linoleoyl (18:2), linolenoyl (18:3), arachidonoyl (20:4), and docosahexaenoyl(22:6);

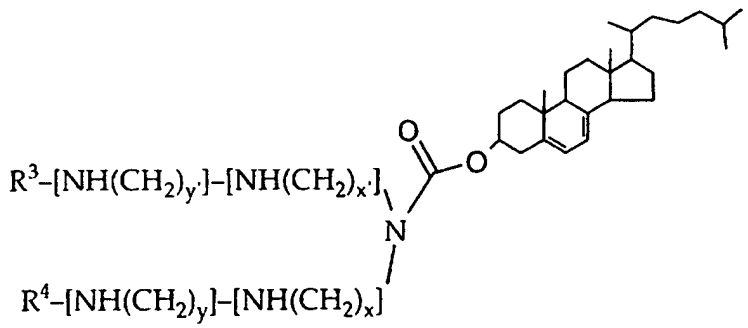
(2) a 1-acyl-2-acyl-sn-glycero-3-phosphoethanolamine having at least one position of unsaturation in each acyl (fatty acid) chain thereof wherein said acyl chains have, individually, from about 12 to about 22 carbon atoms, and are the same or different, and at least one fatty acid chain therein is selected from the group consisting of elaidoyl (18:1 9 trans), and palmitoleoyl (16:1);

(3) a 1-acyl-2-hydroxy-Sn-glycero-3-phosphoethanolamine wherein the fatty acid chain thereof is from about 12 to about 22 carbon atoms, including for example,

palmitoyl (16:0), stearoyl (18:0), and oleoyl (18:1); and

(4) diphytanoyl {16:0 [$(CH_3)4$]} -sn-glycero-3-phosphoethanolamine.

With respect to the cationic amphiphiles that are useful in the practice of the invention, a preferred cationic amphiphile is represented according to the formula



wherein:

each of x, x', y, and y' is a whole number other than 0 or 1, except that, optionally,

the entire term $[NH(CH_2)_{y'}]$ can be absent;

R^3 and R^4 are hydrogen;

the total number of nitrogen and carbon atoms in an

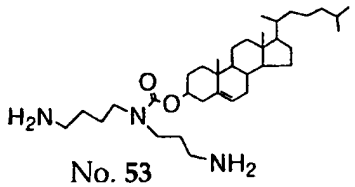
R^3— $[NH(CH_2)_{y'}]$— $[NH(CH_2)_{x'}]$ group, or in an

R^4— $[NH(CH_2)_y]$— $[NH(CH_2)_x]$ group, is less that about 30; and

and wherein, optionally, the double bond at C5, and/or the double bond at C7,

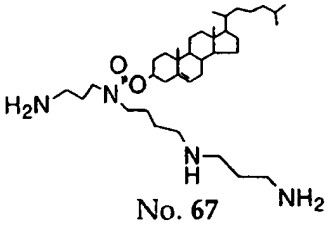
in the steroid ring is hydrogenated.

Specific examples of such amphiphiles include:



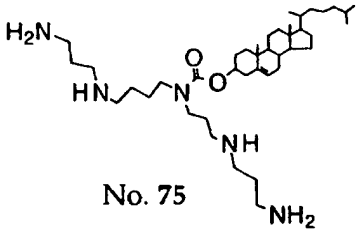
No. 53

N^4-spermidine cholesteryl carbamate



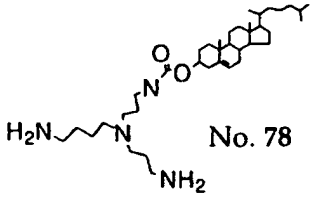
No. 67

N^4-spermine cholesteryl carbamate



No. 75

N^1,N^8-Bis (3-aminopropyl)- N^4 - spermidine cholesteryl carbamate



No. 78

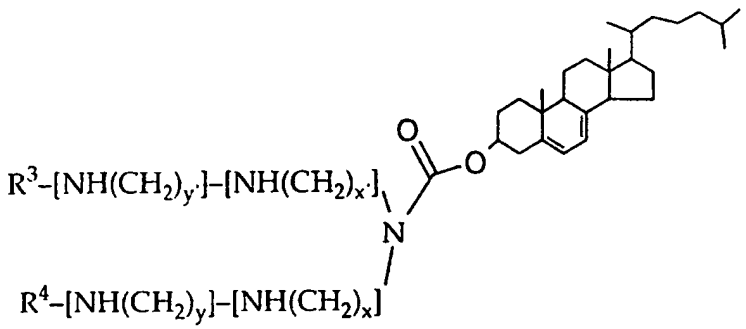
N(N^4-3-aminopropylspermidine) cholesteryl carbamate

A further aspect of the invention involves the discovery that particular neutral co-lipids (termed helper co-lipids) can contribute to the effectiveness of other co-lipids in amphiphile/co-lipid compositions even if such helper co-lipids are relatively ineffective if provided alone, that is, as the only co-lipid to be mixed with the cationic amphiphile(s).

Accordingly, there are provided compositions comprising a solid or

liquid dispersion of:

(A) a cationic amphiphile according to the formula



wherein:

each of x, x', y, and y' is a whole number other than 0 or 1, except that, optionally,

the entire term $[NH(CH_2)_{y'}]$ can be absent;

R^3 and R^4 are hydrogen;

the total number of nitrogen and carbon atoms in an

R^3— $[NH(CH_2)_{y'}]$— $[NH(CH_2)_{x'}]$ group, or in an

R^4— $[NH(CH_2)_y]$— $[NH(CH_2)_x]$ group, is less that about 30; and

and wherein, optionally, the double bond at C5, and/or the double bond at C7,

in the steroid ring is hydrogenated;

(B) a first neutral co-lipid according to the formula 1-acyl-2-acyl-sn-glycero-3-phosphoethanolamine having at least one position of unsaturation in each acyl (fatty acid) chain thereof wherein said acyl chains have, individually, from about 16 to about 18 carbon atoms, and are the same or different; and

(C) at least one helper neutral co-lipid selected from the group consisting of:

(1) a 1-acyl-2-acyl-sn-glycero-3-phosphoethanolamine wherein each acyl chain has from about 16 to about 18 carbon atoms, and wherein said 1-acyl chain is fully saturated, and said 2-acyl chain has at least one position of unsaturation;

(2) a 1-acyl-2-hydroxy-Sn-glycero-3-phosphoethanolamine wherein said acyl group thereof has from about 16 to about 18 carbon atoms; and

(3) a 1-acyl-2-acyl-sn-glycero-3-phosphoethanolamine wherein each acyl chain thereof has from about 16 to about 18 carbon atoms and at least one position of trans-unsaturation, and said acyl chains are the same or different.

The pharmaceutical compositions of the invention may be formulated to contain one or more additional physiologically acceptable substances that stabilize the compositions for storage and/or contribute to the successful intracellular delivery of the biologically active molecules.

In a further aspect, the invention provides a method for facilitating the transfer of biologically active molecules into cells comprising the steps of: preparing a dispersion of one or more cationic amphiphiles and one or more neutral co-lipids, forming a complex between said amphiphile(s) and co-lipid(s) and said therapeutic molecule (for example, a plasmid containing an encoding DNA sequence), and contacting cells with said complex.

Further additional and representative aspects of the invention are described according to the Detailed Description of the Invention which follows directly.

Brief Description of the Drawings

FIGURE1 depicts representative Group I cationic amphophiles.

FIGURE 2 depicts representative steroid lipophilic groups.

FIGURE 3 depicts representative steroid lipophilic groups.

FIGURE 4 depicts a transacylation reaction.

FIGURE 5 depicts representative Group II cationic amphiphiles.

FIGURE 6 depicts representative Group III cationic amphiphiles.

FIGURE 7 depicts representative Group IV cationic amphiphiles.

FIGURE 8 provides a map of pCMVHI-CAT plasmid.

FIGURE 9 depicts the hybrid intron of pCMVHI-CAT.

FIGURE 10 (panel A) provides a map of pCF1/CAT plasmid.

FIGURE 10 (panel B) provides a map of pCF2/CAT plasmid.

FIGURE 11 depicts in vivo CAT assay data for specific lipid 67/neutral co-lipid compositions.

FIGURE 12 depicts in vitro expression data for a reporter gene and certain cationic amphiphile/neutral co-lipid compositions.

FIGURE 13 depicts in vivo expression data for a reporter gene and certain cationic amphiphile/neutral co-lipid compositions.

Detailed Description of the Invention

This invention provides for compositions that are useful to facilitate transport of biologically active molecules into cells. Such a composition comprises one or more biologically active molecules, one or more cationic amphiphiles and one or more neutral co-lipids.

Biological molecules for which transport into cells can be facilitated according to the practice of the invention include, for example, genomic DNA, cDNA, mRNA, antisense RNA or DNA, polypeptides and small molecular weight drugs or hormones. Representative examples thereof are mentioned below in connection with the description of therapeutic (pharmaceutical) compositions of the invention.

In an important embodiment of the invention, the biologically active molecule is an encoding polynucleotide that is expressed when placed in the cells of a patient leading to correction of a metabolic defect. In a particularly important example, the polynucleotide encodes for a polypeptide having an amino acid sequence sufficiently duplicative of that of human cystic fibrosis transmembrane regulator ("CFTR") to allow possession of the biological property of epithelial cell anion channel regulation. Preferably, this polynucleotide encodes human wild type CFTR.

Neutral Co-lipids Useful in the Practice of the Invention

Group I Co-Lipids

It is recognized in the art that neutral co-lipids have properties that compliment those of cationic amphiphiles. Nonetheless, while very extensive research has been conducted in order to identify novel cationic amphiphiles having, for example, high transfecting activity, comparatively little work has been done to develop optimal co-lipids, or optimal cationic amphiphile/co-lipid combinations. Routinely, in fact, dioleoyl-sn- glycero-3-

phosphoethanolamine ("DOPE") has been simply selected as the neutral co-lipid of choice, since it is generally recognized as effective in this role.

According to the practice of the present invention, it has been determined that particular phosphoethanolamines offer improved performance when compared to DOPE. Typically, such neutral co-lipids, according to the formula 1-acyl-2-acyl-sn-glycero-3-phosphoethanolamine, have at least two positions of unsaturation in each acyl (fatty acid) chain thereof wherein said acyl chains have, individually, from about 12 to about 22 carbon atoms, preferrably about 16 to about 18 carbon atoms, and are the same or different. The number of carbon atoms in each acyl chain may be, independently, even or odd. The individual positions of unsaturation may be cis or trans, although generally, cis is preferred over trans. Without being limited as to theory, and using the example of an encoding DNA as the therapeutic molecule, it is believed that the additional degree of unsaturation (compared to DOPE) facilitates beneficial interaction of the co-lipid with the cationic amphiphile (including with a cationic amphiphile/DNA complex), facilitates fusion of therapeutic compositions with target cell plasma membranes, and facilitates one or more intracellular events necessary to further process the encoding polynucleotide, such as escape from an endosomal compartment.

Representative examples of such highly effective 1-acyl-2-acyl-sn-glycero-3-phosphoethanolamines include dilinoleoyl-sn-glycero-3-phospho-ethanolamine-(18:2), dilinolenoyl-sn-glycero-3-phosphoethanolamine(18:3), diarachidonoyl-sn-glycero-3-phosphoethanolamine (20:4), and didocosahexaenoyl-sn-glycero-3-phosphoethanolamine (22:6). Such phospholipids may be purified or synthesized according to methods recognized in the art, and may in many cases be obtained from commercial suppliers, including Avanti Polar Lipids, Alabaster, AL, USA. It is noted also

that the positions of any double bonds need not correspond with double bonds found in phospholipids known to exist in natural sources.

Accordingly, the present invention provides for novel compositions of cationic amphiphiles and neutral co-lipids that, when provided with an expressible polynculeotide, permit expression that is 5 to 10 fold better than that achieved when the same amphiphiles are selcted and DOPE alone is used as co-lipid.

Highly preferred as neutral co-lipids in the practice of the invention are the following co-lipid species: dilinoleoyl-sn-glycero-3-phosphoethanolamine (18:2), dilinolenoyl-sn-glycero-3-phosphoethanolamine (18:3), and the asymmetric species 1-linoleoyl -2-linolenoyl-sn-glycero-3-phosphoethanolamine, and 2-linoleoyl -1-linolenoyl-sn-glycero-3-phosphoethanolamine.

Related to the above preferred neutral co-lipids is an additional class of co-lipids characterized by the formula 1-acyl-2-acyl-sn-glycero-3-phosphoethanolamine, wherein one acyl (fatty acid) chain has only one position of unsaturation, and the other acyl chain has two or more positions of unsaturation, and each such chain has, individually, from about 12 to about 22 carbon atoms. As before, the number of carbon atoms in each acyl chain may be, independently, even or odd, and the individual positions of unsaturation may be cis or trans, although generally, cis is preferred. According to this aspect of the invention, use of the following acyl chains is preferred: linoleoyl (18:2), linolenoyl (18:3), elaidoyl (18:1, 9 trans), oleoyl (18:1, 9 cis), and palmitoleoyl (16:1). According to the practice of the invention, useful species are formed irrespective of whether the singly-unsaturated acyl chain occupies the 1- or 2- position.

Group II Co-Lipids

According to the practice of the invention, it has been determined that

there are additional co-lipids that may be formulated with the co-lipids of Group I, there resulting compositions that are highly effective at facilitating, for example, expression of transfected encoding DNA. The performance that may be achieved thereby is not only improved, but is in certain cases synergistically improved. This is all the more surprising given than certain of the Group II co-lipids may not be particularly active when used as the only co-lipid species.

According to this aspect of the invention, a lipid composition is prepared which consists of two co-lipid species and the cationic amphiphile. It is highly preferred, as it is for the simpler compositions described above, that all the lipid components thereof be in homogeneous intimate contact prior to being resuspended in water for contact with the therapeutic molecule, such as an encoding DNA. Such intimate contact is achieved, for example, by drying down from chloroform or other suitable solvents.

Representative of these co-lipids are:

(1) a 1-acyl-2-acyl-sn-glycero-3-phosphoethanolamine wherein each acyl chain has from about 12 to about 22 carbon atoms, and wherein said 1-acyl chain is fully saturated, and said 2-acyl chain has at least one position of unsaturation, for which representative examples include 1-palmitoyl -2-oleoyl-sn-glycero-3-phosphoethanolamine (designated 16:0,18:1), 1-palmitoyl -2-linoleoyl-sn-glycero-3-phosphoethanolamine, and 1-palmitoyl -2-linolenoyl-sn-glycero-3-phosphoethanolamine (it is noted that the 1-palmitoyl -2-oleoyl-sn-glycero-3-phosphoethanolamine species is effective alone, as the only co-lipid, for use with cationic amphiphiles).

(2) a 1-acyl-2-hydroxy-Sn-glycero-3-phosphoethanolamine wherein said acyl group thereof has from about 12 to about 22 carbon atoms whether saturated or including points of unsaturation, for which representative examples include 1-palmitoyl-2-hydroxy-Sn-glycero-3-phosphoethanolamine (16:0), 1-

stearoyl-2-hydroxy-Sn-glycero-3-phosphoethanolamine(18:0), 1-oleoyl-2-hydroxy-Sn-glycero-3-phosphoethanolamine (18:1), 1-linoleoyl-2-hydroxy-Sn-glycero-3-phosphoethanolamine(18:2), and 1-linolenoyl-2-hydroxy-Sn-glycero-3-phosphoethanolamine(18:3).

Additional Co-Lipids

It has also been determined that additional co-lipids are useful in the practice of the invention, and may be used either in combination with the aforementioned co-lipids of Group I or II, or may be used as the only co-lipid to be added to the cationic amphiphile(s) compositions.

Such additional co-lipids include:

(1) a 1-acyl-2-acyl-sn-glycero-3-phosphoethanolamine wherein each acyl chain thereof has from about 12 to about 22 carbon atoms, preferrably from about 16 to about 18 carbon atoms, and at least one acyl chain has a position of trans-unsaturation, and said acyl chains are the same or different, for which a representative example is dielaidoyl(18:1 9 trans)-sn-glycero-3-phosphoethanolamine;

(2) diphytanoyl (16:0 [$(CH_3)_4$])-sn-glycero -3- phosphoethanolamine; and

(3) dipalmitoleoyl-sn-glycero-3-phosphoethanolamine (16:1), which is particularly effecitve as sole co-lipid, and is effective also in combination with the 1-acyl-2-hydroxy-Sn-glycero-3-phosphoethanolamines described herein.

With respect to all of the types of neutral co-lipids described above, the representation of, for example, well known species with particular locations for positioning of any double bonds along the fatty acid chains is not intended to preclude other structural isomers having similar degrees of unsaturation, whether of cis or trans, but wherein the double bonds are in different positions. Rather, the use of such structural isomers is within the practice of the invention as long as the co-lipids are sufficiently stable.

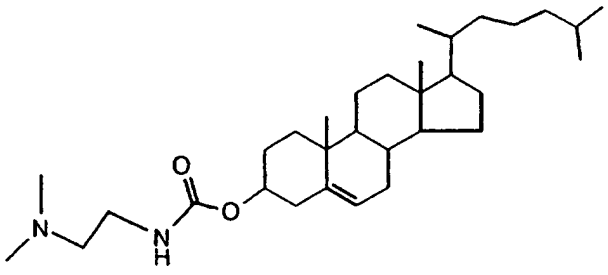
Cationic Amphiphiles Useful in the Practice of the Invention

In connection with the identification of cationic amphiphiles useful in the practice of the present invention, it is noted that "cationic" means that the R groups, as defined herein, tend to have one or more positive charges in a solution that is at or near physiological pH. Such cationic character may enhance interaction of the amphiphile with therapeutic molecules (such as nucleic acids), or with cell structures (such as plasma membrane glycoproteins), thereby contributing to successful entry of the therapeutic molecules into cells, or processing within subcompartments (such as an endosome) thereof. In this regard, the reader is referred to the numerous theories in the art concerning transfection-enhancing function of cationic amphiphiles, none of which is to be taken as limiting on the practice of the present invention.

As aforementioned, any cationic amphiphile that can be shown to facilitate transport of, for example, polynucleotides into cells is useful in the practice of the invention. There are, however, described below particular kinds of cationic amphiphiles that are particularly effective in the practice of the invention.

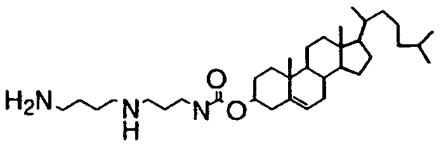
Preferred Cationic Amphiphiles of the Invention

Generally speaking, cationic amphiphiles containing steroid groups are preferred in the practice of the invention. Representative of such amphiphiles is 3ß [N-(N^1,N^1 - dimethylaminoethane)-carbamoyl] cholesterol, commonly known as "DC-chol", as described in U.S. Patent No. 5, 283,185 to Epand et al. The amphiphile is represented as follows:

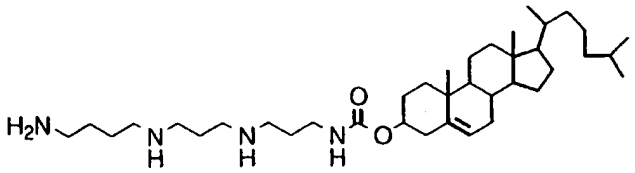


Typically, amphiphiles of this type contain a sterol group connected to an cationic domain, defined by one or more alkyl amine moieties, that can bear at least a partial net positive charge at or near physiological pH. The sterol group and cationic domain are connected via a linker of which many kinds have been described.

Additional amphiphiles of this type include, for example,

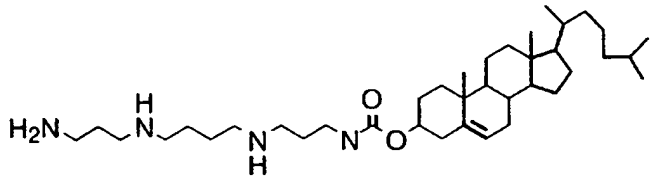


(N^1-spermidine cholesteryl carbamate);



(N^1-Thermospermine cholesteryl carbamate);

and



(N^1-Spermine cholesteryl carbamate).

With respect to the design and orientation of steroid groups of the preferred amphiphles of the invention, the following considerations are of note. Steroids are widely distributed in the animal, microbial and plant kingdoms. They may be defined as solid alcohols that typically contain, as their basic skeleton, 17 carbon atoms arranged in the form of a perhydrocyclopenteno-phenanthrene ring system. Accordingly, such compounds include bile acids, cholesterol and related substances, vitamin D,

certain insect molting hormones, certain sex hormones, corticoid hormones, certain antibiotics, and derivatives of all of the above wherein additional rings are added or are deleted from the basic structure. [see Natural Products Chemistry, K. Nakanashi et al. eds., Academic Press, Inc., New York (1974), volume 1, at Chapter 6 for a further discussion of the broad classes of molecules that are understood in the art to be steroids]. Additionally, for the purposes of the invention, the term steroid is used broadly to include related molecules derived from multiple isoprenoid units, such as vitamin E. Steroids representative of those useful in the practice of the invention are shown in Figures 1, 2, 3 and 5.

As elaborated below, certain preferred amphiphiles of the invention include a steroid component "Z" that is selected from the group consisting of 3-sterols, wherein said sterol molecule is linked by the 3-O- group thereof , or by N- in replacement thereof, to Y (see Figure 1, and below under the heading "Group I Preferred Amphiphiles"). Such structures include, for example, spermidine cholesterol carbamate, spermine cholesterol carbamate, spermidine 7-dehydrocholesteryl carbamate, lysine 3-N-dihydrocholesteryl carbamate, spermidine cholestamine urea, and N-3-aminopropyl-N-4-aminobutylcholestamine.

In a further preferred embodiment, the steroid group is linked to Y (or directly to X if Y is absent) from ring position 17 of the steroid nucleus (see Figures 1 and 3), or from the arm that normally extends from position 17 in many steroids (see Figures 1 and 3), or from any shortened form of said arm.

In connection with the selection of steroids for inclusion in the amphiphiles of the invention, it is preferred that the molecules have structures which can be metabolized by the body and are nontoxic at the doses thereof that are used. Preferred are steroids such as cholesterol and ergosterol that are substantially non toxic and which possess biologically

normal stereospecificity in order to facilitate their safe metabolism in patients. Additional steroids useful in the practice of the invention include, for example, ergosterol B1, ergosterol B2, ergosterol B3, androsterone, cholic acid, desoxycholic acid, chenodesoxycholic acid, lithocholic acid and, for example, various derivatives thereof as are shown in the panels of Figures 2 and 3.

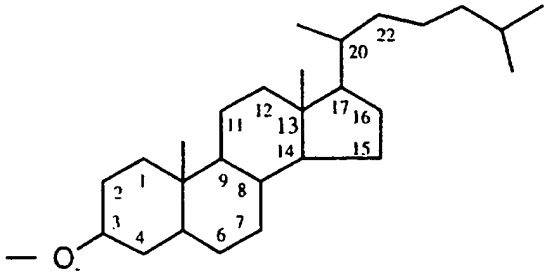
With respect to the orientation of the steroid lipophilic group, that is, how the group is attached(with or without a linker) to the cationic (alkyl) amine groups of an amphiphile, the following further information is of note. Any ring position or substituent on the steroid can in general be used as point of attachment. It is preferred, however, to use a point of attachment that (1) mimimizes the complexity of chemical syntheses, and (2) is positioned near either "end" of the steroid molecule, for example , a position near ring position 3, or near ring position 17(or the arm that typically extends therefrom). Such positions provide an orientation of the steroid with respect to the rest of the amphiphile structure that faciliates bilayer formation, and/or micelle formation, and/or stabilizes interaction with the biologically active molecules to be carried into the target cells. Representative structures showing attachment of the cationic (alkyl) amine groups to the steroid lipophilic group through the arm extending from ring position 17 therof are shown in Figure 3 (panels A, B). With respect to this type of structure, it is further preferred that any polar groups on the steroid, such as may be attached to ring position 3, be either removed or capped (for example, hydroxy as methoxy) to avoid potentially destabilizing bilayer or micelle structures.

The representation in Figure 3 of cationic amphiphiles in which the steroid lipophilic group thereof is linked to the cationic alkylamine groups through steroid ring position 17 is but an example of the invention. Similarly,

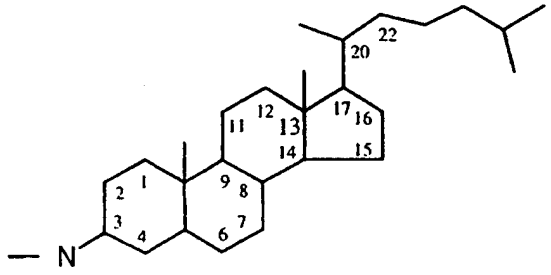
the representation in Figure 1 of cationic amphiphiles in which the steroid lipophilic group thereof is linked to the cationic alkylamine groups through steroid ring position 3 is an example of the invention. As aforementioned, use of any steroid ring position (or moiety or branch extending therefrom) as point of attachment is within the practice of the invention.

Preferred steroids for use as group "Z" according to the practice of the invention include:

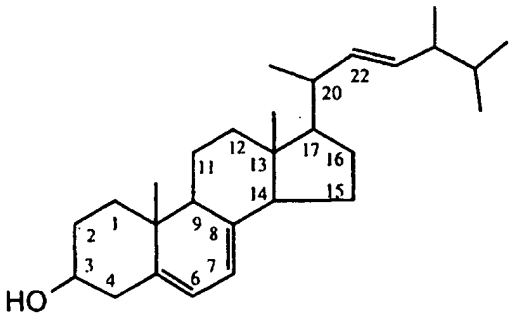
3- sterols (derived from cholesterol)



3-N steryl groups (patterned on cholesterol)



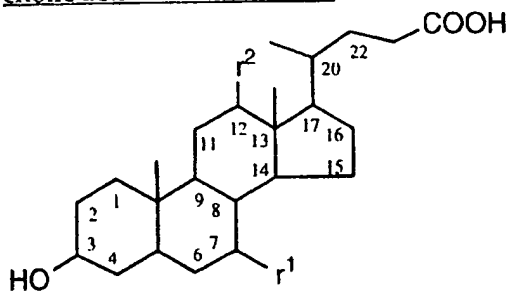
ergosterol and derivatives



Representative species of steroid that are patterened on ergosterol and

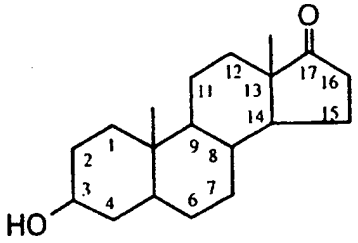
that may be used to define the structure of cationic amphiphiles of the invention include: ergosterol (double bonds as shown); ergosterol B1 (8, 9; 14, 15; 22, 23); ergosterol B1 (6, 7; 8, 14; 22, 23); ergosterol B1 (7, 8; 14, 15; 22, 23); and lumisterol (the 9b-H isomer of ergosterol).

cholic acid and derivatives



Representative species of steroid that are patterened on cholic acid and that may be used to define the structure of cationic amphiphiles of the invention include: cholic acid wherein r^1 and r^2 = OH; desoxycholic acid wherein r^1 = H and r^2 = OH; chenodesoxycholic acid wherein r^1 = OH and r^2 = H; and lithocholic acid wherein r^1 and r^2 = H.

androsterone and derivatives thereof



The most preferred cationic amphiphiles of the present invention contain further distinctive structural features: (1) the presence of a lipophilic group which is connected directly, or through a linking group, to two cationic groups (see below) that themselves comprise amino, alkylamine or polyalkylamine groups, there resulting an overall and novel "T-shaped"

structure; and (2) in many cases, and in comparison with numerous art-recognized amphiphiles, the use of a relatively short linking group to bring into close proximity the lipophilic and cationic regions of the amphiphile. Without being limited as to theory, it is believed that these features contribute substantially to the transfection-enhancing capability of these compounds. The effectiveness of the compositions of the invention thus arises from the selection of particular cationic amphiphiles and also through the selection of particularly useful neutral co-lipids or combinations thereof. Preferred amphiphiles are represented in Groups I, II, III, and IV as described below.

Characteristic and novel features of these preferred amphiphiles of the invention include first, that the linking group that connects the two cationic amine groups to the lipophilic group is preferrably short, or absent entirely, and second, that the resultant linking of the the two cationic R groups to the lipophilic group forms a T-shaped structure when viewed from the position of atom "X" (a carbon or nitrogen atom) as depicted below, for example, in Structures (I), (II), (III) and atom "E" in Structure (IV).

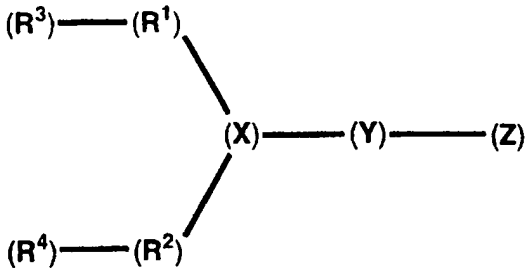
Applicants have also noted that numerous of the cationic amphiphiles of the invention have structural features in common with naturally occurring polyamines such as spermine and spermidine (including N-atom spacing). In this regard, the structures of amphiphiles 53, 67, 78, 90, and 91 are representative. It has been determined that the placement of the nitrogen atoms in the polar head groups of the amphiphiles such that they are separated by one or more combinations of 3 and 4 carbon atoms leads to high in vivo transfection efficiency for plasmid transgenes complexed therewith. Applicants have also noted that these in-common structural features may have a useful effect upon the binding of the amphiphiles to DNA, and on interaction with cell surface polyamine receptors. Interaction with cell polyamine receptors may be particularly important with respect to the

treatment of cancer cells by gene therapy, since the DNA replication requirements of such cells may lead to high level expression of such receptors.

Group I Preferred Amphiphiles

In connection with the design of the Group I amphiphiles of the invention, the following considerations are of note. Many of these design features are then discussed in connection with the other amphiphiles of the invention, those classified under Groups II, II and IV.

Accordingly, there are provided cationic amphiphiles of Group I (see Figure 1, panels A , B, and C) capable of facilitating transport of biologically active molecules into cells, said amphiphiles having the structure (I),



(I)

wherein:

Z is a steroid;

X is a carbon atom or a nitrogen atom;

Y is a short linking group , or Y is absent;

R^3 is H, or a saturated or unsaturated aliphatic group;

R^1 is --NH-- , an alkylamine, or a polyalkylamine;

R^4 is H, or a saturated or unsaturated aliphatic group;

R^2 is --NH-- , an alkylamine, or a polyalkylamine;

and wherein R^1 is the same or is different from R^2 , except that both R^1 and R^2 cannot be --NH--.

The Linking Group

Preferably the linking group that connects the lipophilic group to the two cationic R groups is relatively short. It is preferred that within linking group Y are contained no more than about three or four atoms that themselves form a bridge of covalent bonds between X and Z. Examples of Y groups include --

$-(CH_2)_2-$; $-(CH_2)_3-$; $-(CH_2)-(C=O)-$; $-(CH_2)n-NH-(C=O)-$ where n is preferably 4 or less. Additional linking groups useful in the practice of the invention are those patterned on small amino acids such as glycinyl, alanyl, beta-alanyl, serinyl, and the like.

With respect to the above representations, the left hand side thereof-as depicted- is intended to bond to atom "X", and the right hand side thereof to group "Z"(see structure I).

In certain preferred embodiments of the invention, Y is a linking group wherein no more than one atom of this group forms a bond with both "X"and "Z". Examples of preferred linking groups include $--CH_2--$, >C=S, and >C=0. Alternatively, the linking group "Y"may be absent entirely.

As aforementioned (see Structure I, directly above), "X" forms a connecting point in the amphiphiles to which is also attached the two cationic R groups. As can be seen therein (see also Figure 1), the placement of the nitrogen atom that represents "X" clearly causes the molecule to assume a T-shape.

Steroid Lipophilic Groups

The steroid lipophilic groups of these amphiphiles have been described above.

Selection of Groups R^1, R^2, R^3, and R^4

For $R^3$and R^4:

According to the practice of the invention R^3 and R^4 are,

independently, H, or saturated or unsaturated aliphatic groups. The aliphatic groups can be branched or unbranched. Representative groups include alkyl, alkenyl, and cycloalkyl.

For R^1 and R^2:

R^1 and R^2 represent structures recognized in the art as being amine; alkylamines (including primary, secondary, and tertiary amines), or extended versions thereof-herein termed "polyalkylamines". It is understood that both alkylamine and polyalkylamine groups as defined herein may include one or more carbon-carbon double bonds and the use of such alkenylamines is therefore within the practice of the invention.

Representative alkylamines include: (a) -- $NH-(CH_2)_z$ -- where z is other than 0; (b) -- $[[CH_3(CH_2)_y]N]-(CH_2)_z$ -- where z is other than 0; and (c) -- $[[CH_3(CH_2)_x][CH_3(CH_2)_y]]N-(CH_2)_z$ -- where z is other than 0.

With respect to the circumstance where one or both of R^1 and R^2 are tertiary amines, such as is represented in Structure (c) above, it is understood that a hydrogen atom corresponding to either R^3 or R^4, as appropriate, may or may not be present since such hydrogen atoms correspond to the N:H(+) structure whose level of protonation will vary according to pH.

The term "polyalkylamine" as referred to herein defines a polymeric structure in which at least two alkylamines are joined. The alkylamine units that are so joined may be primary or secondary, and the polyalkylamines that result may contain primary, secondary, or tertiary N-atoms. The alkylamine (sub)units may be saturated or unsaturated, and therefore the term "alkylamine" encompasses alkenylamines in the description of the invention.

Representative resultant polyalkylamines include: (d) -- $[NH-(CH_2)(z)]_q$ -- , where z is other than 0, and q is 2 or higher; (e) -- $[NH-(CH_2)(y)]_p$ -- $[NH-(CH_2)(z)]_q$ -- , where y and z are each other than 0, and p and q are each other than 0; (f) -- $[NH-(CH_2)(x)]_n$ --$[NH-(CH_2)(y)]_p$ -- [NH-

$(CH_2)(z)]_q$ -- , where x, y, and z are each other than 0, and n, p and q are each other than 0; (g) -- $[NH\text{-}(CH_2)(w)]_m$ -- $[NH\text{-}(CH_2)(x)]_n$ --$[NH\text{-}(CH_2)(y)]_p$ -- $[NH\text{-}(CH_2)(z)]_q$ -- , where w, x, y, and z are each other than 0, and m, n, p, and q are each other than 0; (h) -- $[NH\text{-}(CH_2)(w)]_m$ -- $[NH\text{-}(CH_2)(x)]_n$ --$[[CH_3(CH_2)_y]N]\text{-}(CH_2)_z$ -- , where x, n and z are each other than 0; (i) -- $[NH\text{-}(CH_2)(w)]_p$ -- $[[CH_3(CH_2)_x]N]\text{-}(CH_2)_y$ -- $[NH\text{-}(CH_2)(z)]_q$ -- , where w, p, y, z, and p are each other than 0; and (j) -- $[NH\text{-}(CH_2)(v)]_l$ -- $[NH\text{-}(CH_2)(w)]_m$ -- $[NH\text{-}(CH_2)(x)]_n$ --$[NH\text{-}(CH_2)(y)]_p$ -- $[NH\text{-}(CH_2)(z)]_q$ -- , where v, w, x, y, and z are each other than 0, and l, m, n, p, and q are each other than 0.

As mentioned above R^1 and R^2, independently, can be -- NH-- , an alkylamine, or a polyalkylamine, and can be the same or different from each other, except that both R^1 and R^2 cannot be --NH-- in order to (1) preserve the "T- shape" of the resultant compound, and (2) to provide for the stability thereof. It is preferred that - in combination- the combined backbone length of R^3R^1 (or of R^4R^2) be less than about 40 atoms of nitrogen and carbon, more preferrably less than about 30 atoms of nitrogen and carbon.

In the case where the R^1 group adjacent to R^3 (or R^2 adjacent to R^4) includes a terminal nitrogen atom that defines a tertiary center, then a quaternary amine is formed (at that nitrogen atom of R^1) if R^3 is an aliphatic group, and a tertiary amine remains (at that nitrogen atom of R^1) if R^3 is H. Accordingly, with respect to such resultant R^3R^1 or R^4R^2 structures, representative respective formulas are:

(k) $H\text{-}(CH_2)(w)$ —$[[CH_3(CH_2)_x][CH_3(CH_2)_y]N]\text{-}(CH_2)_z$ — , where w and z are each other than zero; and (l) H — $[[CH_3(CH_2)_x][CH_3(CH_2)_y]N]\text{-}(CH_2)_z$ — , where z is other than zero.

In connection with interpreting the structural diagrams described herein, it is intended that the attachment of R^3R^1— (or R^4R^2—) structures to

atom "X" is through the right hand side (as depicted) of the R^3R^1—, that is, through a CH_2— moiety. The coefficents (i.e. v, w, x, y, and z and l, m, n, p, and q) as depicted herein represent whole numbers. For the purposes of the invention, "whole number" means 0 and the natural numbers 1,2,3,4,5,6.....and up, unless specifically restricted.

With respect to the amphiphiles of the invention including those represented by formulas (a) to (l), it is noted that there are certain preferences concerning the design of such groups depending on whether atom 'X" as it is shown according to structure (I) above, is a nitrogen atom or a carbon atom. If "X" is nitrogen, then amphiphiles containing R^3-R^1 (or R^4-R^2) groups that end in an N atom [i.e formula (e) where z equals 0 and q=1; formula (h) where z equals 0] are not preferred, since the resultant N-N linkage involving position X results in an amphiphile that may be unstable and/or difficult to prepare. An additional group of structures that are difficult to prepare and/or are unstable is represented, for example, by the R sequence (whether in R^1, or bridging R^1 and R^3) --NH- CH_2-NH-CH_2-- . Accordingly, use of such structures [i.e. formula (a) where Z equals 1, formula (e) where one or both of y and z equals 1]in the practice of the invention is not preferred.

With respect to the design of structures (such as those depicted above) for inclusion in cationic amphiphiles, the following further considerations are of note. Any combination of alternating amine and alkyl moieties creates an R structure within the scope of the invention. A polyalkylamine may be represented, for example, by the formulas above, although many more structures (such structures being within the scope of the invention) can be depicted by extending the number of, or types or combinations of, alkylamine subunits within the amphiphile structure. That further such variations can be made is apparent to those skilled in the art.

It is noted that a polyalkylamine group (or resultant R^3R^1 group) that

is very long may interfere, for example, with the solubility of the resultant amphiphile, or interfere with its ability to stably interact with the biologically active molecule selected for intracellular delivery. In this regard, polyalkylamines (or resultant R^3R^1 groups) having a backbone length of about 40 nitrogen and carbon atoms, or more, may not be suitable for inclusion in amphiphiles. However, for each such proposed structure, its properties may be determined by experimentation, and its use is nonetheless within the practice of the invention.

Accordingly, specific alkylamine and polyalkylamine structures result as follows:

Table 1

For R^1 and/or R^2

(1) -NH-

(2) -NH-$(CH_2)_{(2)}$-

(3) -NH-$(CH_2)_{(3)}$-

(4) -NH-$(CH_2)_{(4)}$-

(5) -NH-$(CH_2)_{(6)}$-

(6) -NH-$(CH_2)_{(3)}$- NH-$(CH_2)_{(4)}$-

(7) -NH-$(CH_2)_{(2)}$- NH-$(CH_2)_{(2)}$-

(8) -NH-$(CH_2)_{(4)}$- NH-$(CH_2)_{(3)}$-

(9) -NH-$(CH_2)_{(y)}$- NH-$(CH_2)_{(z)}$-

(10) -NH-$(CH_2)_{(x)}$-NH-$(CH_2)_{(y)}$-NH-$(CH_2)_{(z)}$-

(11) -NH-$(CH_2)_{(w)}$-NH-$(CH_2)_{(x)}$-NH-$(CH_2)_{(y)}$-NH-$(CH_2)_{(z)}$-

(12) -NH-$(CH_2)_{(v)}$ -NH-$(CH_2)_{(w)}$-NH-$(CH_2)_{(x)}$-NH-$(CH_2)_{(y)}$-NH-$(CH_2)_{(z)}$-

(13) -[NH-$(CH_2)_{(w)}]_m$ -- [NH-$(CH_2)_{(x)}]_n$ --[[$CH_3(CH_2)_y$]N] -$(CH_2)_z$ -

(14) -[NH-$(CH_2)_{(x)}]_n$ --[[$CH_3(CH_2)_y$]N] -$(CH_2)_z$ -

(15) -[NH-$(CH_2)_{(w)}]_m$ -- [NH-$(CH_2)_{(x)}]_n$ --[[$CH_3(CH_2)_y$]N] -$(CH_2)_z$ -

(16) - [[$CH_3(CH_2)_x$][$CH_3(CH_2)_y$]N]-$(CH_2)_z$ --

(17) -NH-$(CH_2)_{(z)}$- NH --

(18) -NH-$(CH_2)_{(y)}$-NH-$(CH_2)_{(z)}$-NH --

(19) -NH-$(CH_2)_{(y)}$-CH=CH-$(CH_2)_z$ --

(20) -- [NH-$(CH_2)_{(w)}]_p$ --[[$CH_3(CH_2)_x$]N] -$(CH_2)_y$ -- [NH-$(CH_2)_{(z)}]_q$ -

For R^3 and/or

R^4

(1) H--

(2) CH_3--

(3) CH_3-$(CH_2)_2$--

(4) CH_3-$(CH_2)_4$--

(5) CH_3-$(CH_2)_z$--

(6) CH_3-[CH_3-$(CH_2)_z$]CH --

(7) CH_3-[CH_3-$(CH_2)_2$]CH --

(8) CH_3-[[CH_3-$(CH_2)_y$][CH_3-$(CH_2)_z$]]C --

(9) CH_3-$(CH_2)_z$-CH=CH-CH_2 --

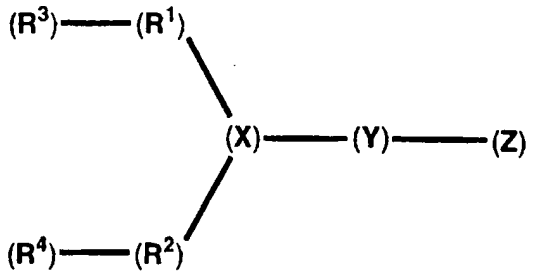
(10) CH_3-[CH_3-$(CH_2)_y$-CH=CH-$(CH_2)_z$]CH --

(11) CH_3-[[CH_3-$(CH_2)_w$-CH=CH-$(CH_2)_x$][CH_3-$(CH_2)_y$-CH=CH-$(CH_2)_z$]]CH --

(12) CH_3-[CH_3-$(CH_2)_y$]CH-$(CH_2)_z$ --

Group II Amphiphiles

Additionally there are provided cationic amphiphiles of Group II (see Figure 5) capable of facilitating transport of biologically active molecules into cells said amphiphiles having the structure (II),



(II)

wherein:

Z is a steroid;

X is a carbon atom or a nitrogen atom;

Y is a linking group or Y is absent;

R^3 is an amino acid, a derivatized amino acid, H or alkyl;

R^1 is --NH-- , an alkylamine, or a polyalkylamine;

R^4 is an amino acid, a derivatized amino acid, H or alkyl;

R^2 is --NH-- , an alkylamine, or a polyalkylamine;

and wherein R^1 is the same or is different from R^2 , except that both R^1 and R^2 cannot be --NH--.

Representative amphiphiles provided according to Group II include amphiphiles 87, 91, 93, 95, 97, 99, 100, and 103. With respect to the structural features of these amphiphiles, and the other amphiphiles of Group II, the following should be considered.

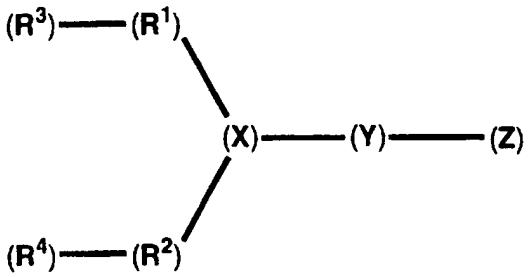
The steroid group may be selected according to the criteria defined above for the Group I amphiphiles. Accordingly, preferred amphiphiles include those selected from 3- sterols, wherein the sterol molecule is linked by the 3-O- group thereof, or by N in replacement thereof, to "Y".

The linking group Y of the Group II amphiphiles consists of an N-acylamino acid (or a derivative thereof), or consists of a group (such as > C=O or > C=S) wherein no more than one atom of said group forms a bond with both "X" and "Z". Optionally, group Y may be absent. Representative N-acylamino groups include an N-Acyl serine (No. 87), an N-Acyl glycine (No. 91), and an N-Acyl aspartic acid (No. 103). With respect to the use of N-Acyl aspartic acid in amphiphile No. 103, it is noted that, as provided, the gamma carboxyl thereof is further derivatized to an additional alkylamine moiety.

The crtiteria for selection of R^1 and R^2 are as set forth for the Group I amphiphiles. R^3 and R^4 represent H or alkyl, or may be natural or artificial amino acids including derivatives of either. Representative examples of R^3 or R^4 amino acid groups include those derived from tryptophan (No. 97) and from arginine (No. 95).

Group III Amphiphiles

Additionally there are provided cationic amphiphiles of Group III (see Figure 6) capable of facilitating transport of biologically active molecules into cells said amphiphiles having the structure (III),



(III)

wherein:

Z is an alkylamine or a dialkylamine, linked by the N-atom thereof, to Y, or directly to X if Y is absent, wherein if Z is a dialkylamine, the alkyl groups thereof can be the same or different;

X is a carbon atom or a nitrogen atom;

Y is a short linking group , or Y is absent;

R^3 is H, or a saturated or unsaturated aliphatic group;

R^1 is --NH-- , an alkylamine, or a polyalkylamine;

R^4 is H, or a saturated or unsaturated aliphatic group;

R^2 is --NH-- , an alkylamine, or a polyalkylamine;

and wherein R^1 is the same or is different from R^2 , except that both R^1 and R^2 cannot be --NH--.

Representative cationic amphiphiles according to the practice of the invention that contain an alkyl amine or dialkylamine as lipophilic group include, for example, N,N-dioctadecyllysineamide; N^1, N^1-dioctadecyl-1,2,6-triaminohexane; N,N-didodecyllysineamide; N,N- didecyllysineamide; spermidine- N,N- dioctadecyl urea; N-myristyllysineamide; and N-(dioctyldecylaminoethyl)-lysineamide . Representative amphiphiles are

depicted (Figure 6) as amphiphiles 43, 47, 56, 60, and 73. With respect to the structural features of these amphiphiles, and the other amphiphiles of Group III, the following should be considered.

With respect to the selection of the lipophilic alkylamine or dialkylamine group "Z", Table 2 below provides representative structures.

Table 2

For "Z"

---

(1) CH_3-$(CH_2)_{13}$-NH--

(2) CH_3-$(CH_2)_z$-NH--

(3) [[$CH_3(CH_2)_{17}$][$CH_3(CH_2)_{17}$]]N--

(4) [[$CH_3(CH_2)_{11}$][$CH_3(CH_2)_{11}$]]N--

(5) [[$CH_3(CH_2)_9$][$CH_3(CH_2)_9$]]N--

(6) [[$CH_3(CH_2)_x$][$CH_3(CH_2)_y$]]N--

(7) [[$CH_3(CH_2)_x$][$CH_3(CH_2)_yCH{=}CH(CH_2)_z$]]N--

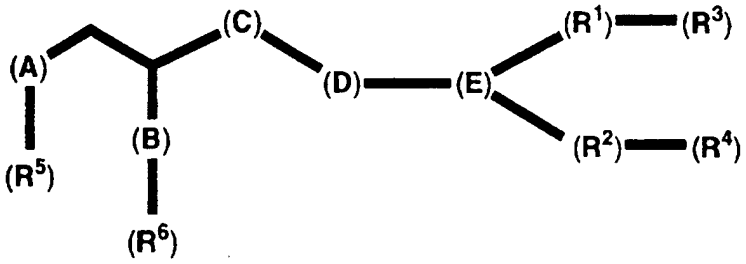
(8) [[$CH_3(CH_2)_w$][$CH_3(CH_2)_xCH{=}CH(CH_2)_yCH{=}CH(CH_2)_z$]]N--

In connection with the selection of suitable alkylamine or dialkylamine groups for inclusion at position Z in the amphiphiles of the invention, an alkyl chain(s) of the group should not be so large in molecular weight that it interferes with the solubility of the amphiphile, or interferes with its ability to interact with plasmid DNA. Additionally, an alkyl chain of an alkylamine or dialkylamine may include one or more points of unsaturation.

The selection of R groups R^1, R^2, R^3, and R^4 follows that disclosed for the Group I amphiphiles, and these R groups may be selected, for example, from Table I. Linking group Y may be seleected as for the Group I amphiphiles, and preferred examples thereof include $-CH_2-$, and > C=O.

Group IV Amphiphiles

Additionally there are provided cationic amphiphiles of Group IV (see Figure 7) capable of facilitating transport of biologically active molecules into cells said amphiphiles having the structure (IV),



(IV)

wherein:

A and B are independently O, N or S;

R^5 and R^6 are independently alkyl or acyl groups and may be saturated or contain sites of unsaturation;

C is selected from the group consisting of $-CH_2-$, >C=O, and >C=S;

E (analogous to "X" in structures I, II, III) is a carbon atom or a nitrogen atom;

D is a linking group such as -NH(C=O)- or -O(C=O)- , or D is absent;

R^3 is H, or a saturated or unsaturated aliphatic group;

R^1 is --NH-- , an alkylamine, or a polyalkylamine;

R^4 is H, or a saturated or unsaturated aliphatic group;

R^2 is --NH-- , an alkylamine, or a polyalkylamine;

and wherein R^1 is the same or is different from R^2 , except that both R^1 and R^2 cannot be --NH--.

Representative amphiphiles of Group IV include Nos. 64, 76, 85, 89, 94, 98, 102, 105, 110, and 111. With respect to the structural features of these amphiphiles, and the other amphiphiles of Group IV, the following should be considered.

With respect to the selection of R^1, R^2, R^3, and R^4, the teachings provided for Group I, II, and III amphiphiles are applicable. As aforementioned, group "E" represents a carbon atom or a nitrogen atom.

R^5 and R^6 are independently alkyl or acyl groups, preferrably containing about 8 to about 30 carbon atoms, and such groups may contain one or more points of unsaturation.

With respect to the selection of Group D, linkers such as -NH(C=O)- or -O(C=O)- are preferred, and are depicted such that the left side thereof in intended to bond to "C" and the right side thereof is intended to bond to "E". Optionally, group D may be absent (amphiphile No.94). Additional linkers may be selected based on the teachings provided with respect to Groups I, II, and III above, and based upon the in vivo test date derived (Figure 15), it is preferred that the linker D be short or absent.

Transacylation Reactions

Although heretofore unrecognized in the art, it has been determined also that certain co-lipids may react chemically with certain types of cationic amphiphiles under conditions of co-storage, there resulting new molecular species. Generation of such new species is believed to occur via mechanisms such as transacylation. In this regard, see Figure 4 which depicts a transacylation reaction involving spermine cholesterol carbamate(No.67) and DOPE, there resulting lyso PE species and multiple forms of particular acyl-cationic amphiphile (designated No. 80).

With respect to such reactions, the following remarks are of interest. With respect to use of amphiphile No.67, it has been observed that a mixture of amphiphile and DOPE, in chloroform solvent, does not appear to participate in such reactions. However, preparing the amphiphile and co-lipid in an aqueous solution where bilayer-containing structures such as liposomes can form will permit transacylation. Additionally, if amphiphile and co-lipid

are dried down to a thin film, such as from chloroform (thereby placing the 2 species in intimate contact), then transacylation also occurs, possibly as a result of entropic effects. It is expected that these phenomena would also apply to lyophilized amphiphile/DOPE preparations.

Accordingly, it is highly preferred to maintain such amphiphile /DOPE preparations at very cold temperatures, such as -70 degrees C. Preparation of amphiphile No. 67 as a mono, di, or tri acetate salt has also been determined to slow transacylations.

It is to be understood that therapeutically-effective pharmaceutical compositions of the present invention may or may not contain such transacylation byproducts, or other byproducts, and that the presence of such byproducts does not prevent the therapeutic use of the compositions containing them. Rather use of such compositions is within the practice of the invention, and such compositions and the novel molecular species thereof are therefore specifically claimed. It is suggested that the practitioner of the art monitor any compositions prepared for reactions similar to those described.

Preparation of Pharmaceutical Compositions and Administration Thereof

The present invention provides for pharmaceutical compositions that facilitate intracellular delivery of therapeutically effective amounts of biologically active molecules. Pharmaceutical compositions of the invention facilitate entry of biologically active molecules into tissues and organs such as the gastric mucosa, heart, lung, and solid tumors. Additionally, compositions of the invention facilitate entry of biologically active molecules into cells that are maintained in vitro , such as in tissue culture. The amphiphilic nature of the cationic amphiphiles used in the practice of the invention enables them to associate with the lipids of cell membranes, other cell surface molecules, and tissue surfaces, and to fuse or to attach thereto. One type of structure that can be formed by amphiphiles is the liposome, a vesicle formed into a more or

less spherical bilayer, that is stable in biological fluids and can entrap biological molecules targeted for intracellular delivery. By fusing with cell membranes, such liposomal compositions permit biologically active molecules carried therewith to gain access to the interior of a cell through one or more cell processes including endocytosis and pinocytosis. However, unlike the case for many classes of amphiphiles or other lipid-like molecules that have been proposed for use in therapeutic compositions, the cationic amphiphiles of the invention need not form highly organized vesicles in order to be effective, and in fact can assume (with the biologically active molecules to which they bind) a wide variety of loosely organized structures. Any of such structures can be present in pharmaceutical preparations of the invention and can contribute to the effectivenesss thereof. As aforementioned, such structures can be beneficially modified by the presence of one or more neutral co-lipids.

Biologically active molecules that can be provided intracellularly in therapeutic amounts using the amphiphile/co-lipid compositions of the invention include:

(a) polynucleotides such as genomic DNA, cDNA, and mRNA that encode for therapeutically useful proteins as are known in the art,

(b) ribosomal RNA;

(c) antisense polynucleotides, whether RNA or DNA, that are useful to inactivate transcription products of genes and which are useful, for example, as therapies to regulate the growth of malignant cells; and

(d) ribozymes.

Representative of the types of low molecular weight biologically active molecules that can be delivered include hormones and antibiotics. In general, and owing, for example, to the potential for leakage or dissociation of contents, vesicles and other structures formed from numerous of the cationic

amphiphiles are not preferred by those skilled in the art in order to deliver low molecular weight biolgically active molecules. Although, not a preferred embodiment of the present invention, it is nonetheless within its practice to deliver such low molecular weight molecules intracellularly.

Cationic amphiphile species of the invention may be blended so that two or more species thereof are used, in combination, to facilitate entry of biologically active molecules into target cells and/or into subcellular compartments thereof. Cationic amphiphiles of the invention can also be blended for such use with amphiphiles that are known in the art.

Dosages of the pharmaceutical compositions of the invention will vary, depending on factors such as half-life of the biologically-active molecule, potency of the biologically-active molecule, half-life of the amphiphile(s), any potential adverse effects of the amphiphile(s) or of degradation products thereof, the route of administration, the condition of the patient, and the like. Such factors are capable of determination by those skilled in the art.

A variety of methods of administration may be used to provide highly accurate dosages of the pharmaceutical compositions of the invention. Such preparations can be administered orally, parenterally, topically, transmucosally, or by injection of a preparation into a body cavity of the patient, or by using a sustained-release formulation containing a biodegradable material, or by onsite delivery using additional micelles, gels and liposomes. Nebulizing devices, powder inhalers, and aerosolized solutions are representative of methods that may be used to administer such preparations to the respiratory tract.

Additionally, the therapeutic compositions of the invention can in general be formulated with excipients (such as the carbohydrates lactose, trehalose, sucrose, mannitol, maltose or galactose) and may also be lyophilized (and then rehydrated) in the presence of such excipients prior to

use. Conditions of optimized formulation for each amphiphile of the invention are capable of determination by those skilled in the pharmaceutical art. By way of example, for spermidine cholesterol carbamate (amphiphile No. 53), it has been determined that use of sucrose is preferred over mannitol in order to prevent formation of amphiphile/DNA aggregates, particularly as the concentration of DNA is increased therein. Addition of such excipients maintains the consistency of lyophilized pharmaceutical compositions during storage, and prevent difficulties such as aggregation, or insolubity, that may likely occur upon rehydration from the lyophilized state.

Accordingly, a principal aspect of the invention involves providing a composition that comprises a biologically active molecule (for example, a polynucleotide) and one or more cationic amphiphiles (including optionally one or more co-lipids), and then maintaining said composition in the presence of one ore more excipients as aforementioned, said resultant composition being in liquid or solid (preferably lyophilized) form, so that: (1) the therapeutic activity of the biologically active molecules is substantially preserved; (2) the transfection-enhancing nature of the amphiphile(or of amphiphile/ DNA complex) is maintained. Without being limited as to theory, it is believed that the excipients stabilize the interaction (complexes)of the amphiphile and biologically active molecule through one or more effects including:

(1) minimizing interactions with container surfaces,

(2) preventing irreversible aggregation of the complexes, and

(3) maintaining amphiphile/DNA complexes in a chemically-stable state, i.e., preventing oxidation and/or hydrolysis.

Although the presence of excipients in the pharmaceutical compositions of the invention stabilizes the compositions and faciliates storage and manipulation thereof, it has also been determined that moderate

concentrations of numerous excipients may interfere with the transfection-enhancing capability of pharmaceutical formulations containing them. In this regard, an additional and valuable characteristic of the amphiphiles of the invention is that any such potentially adverse effect can be minimized owing to the greatly enhanced in vivo activity of the amphiphiles of the invention in comparison with amphiphilic compounds known in the art. Without being limited as to theory, it is believed that osmotic stress (at low total solute concentration) may contribute positively to the successful transfection of polynucleotides into cells in vivo . Such a stress may occur when the pharmaceutical composition, provided in unbuffered water, contacts the target cells. Use of such otherwise preferred compositions may therefore be incompatible with treating target tissues that already are stressed, such as has damaged lung tissue of a cystic fibrosis patient. Accordingly, and using sucrose as an example, selection of concentrations of this excipient that range from about 15 mM to about 200 mM provide a compromise betweeen the goals of (1) stabilizing the pharmaceutical composition to storage and (2) mimizing any effects that high concentrations of solutes in the composition may have on transfection performance.

Selection of optimum concentrations of particular excipients for particular formulations is subject to experimentation, but can be determined by those skilled in the art for each such formulation.

An additional aspect of the invention concerns the protonation state of the cationic amphiphiles of the invention prior to their contacting plasmid DNA in order to form a therapeutic composition. It is within the practice of the invention to utilize fully protonated, partially protonated, or free base forms of the amphiphiles in order to form such therapeutic compositions. Generally it is preferred, though not required, to provide the one or more neutral co-lipids as zwitterions. With respect to amphiphile No. 67 (spermine

cholesterol carbamate), it has been observed that when providing this amphiphile for a transfecting composition with DOPE (itself provided as a zwitterion), transgene expression was best for the free base, but decreased if the amphiphile was prepared as an acetate salt. Activity decreased step-wise through the mono and di acetate salts and was minimal for the tri-acetate salt. Under the circumstances described, the plasmid DNA provided for contacting with the amphiphile was prepared (without buffer) as a sodium salt in water.

Methods of Syntheses

The following methods illustrate production of certain cationic amphiphiles useful in the practice of the invention. Those skilled in the art will recognize other methods to produce these or other amphiphiles.

(A) N^4-Spermidine cholesteryl carbamate

Spermidine cholesterol carbamate (Figure 1, No. 53) was synthesized according to the following procedure which is outlined in Figure 8.

Synthesis of N^1, N^8-DiCBZ -N^4-Spermidine Cholesterol Carbamate

N^1, N^8 - dicarbobenzoxyspermidine (61% yield, m.p. 104 - 105° C) was prepared according to the procedure of S. K. Sharma, M. J. Miller, and S. M. Payne, J. Med. Chem., 1989, 32, 357-367. The N^1, N^8 - dicarbobenzoxyspermidine (25 g , 60.5 mmol) and triethylamine (25 ml, 178 mmol) were dissolved in 625 ml of anhydrous methylene chloride, cooled to 0 - 4°C and stirred under N_2. Cholesteryl chloroformate (27.2 g, 60.6 mmol) was dissolved in 250 ml of methylene chloride and added to the reaction over a 20 minute period. A white precipitate formed upon addition. After the addition was complete, the reaction was stirred at 0 - 4°C for 10 minutes and then at room temperature for 1.5 hr. At this point, the white precipitate completely dissolved. The reaction was followed by TLC with hexane / ethyl acetate 6 / 4 as eluent (product Rf = 0.25). To this reaction mixture was added 625 ml of methylene chloride and 625 ml of water. The layers were then allowed to separate. The organic layer was dried over $MgSO_4$ and filtered. The filtrate was concentrated *in vacuo* to give an oil. Vacuum drying was then carried out overnight. This crude product had a glue-like consistency. The crude product was purified by column chromatography (2 kg silica gel, eluent - hexane / ethyl acetate 6 / 4) to give 46.8 g of the 3-ß-[N^4-(N^1,N^8-dicarbobenzoxyspermidine)carbamoyl] cholesterol (also described herein as N^1, N^8- diCBZ-N^4- spermidine cholesterol carbamate) in 93% yield.

Final Synthesis of Spermidine Cholesterol Carbamate

To 6.0 grams of 10% palladium on activated carbon under N_2 was added a solution of 30 grams of 3-ß-[N^4-(N^1,N^8-dicarbobenzoxyspermidine)carbamoyl] cholesterol in 1 liter of ethanol, see Figure 13. The reaction mixture was purged with N_2 and stirred under H_2 (atmospheric pressure) for 18 hr. The mixture was again purged with N_2 and filtered through a 10 g bed of celite. The filter cake was washed with 2 liters of 10% triethylamine in ethanol and the combined filtrates were concentrated *in vacuo* to a gel. The product was then dried under vacuum overnight to a sticky solid. This crude product was purified by column chromatography (2 kg of silica gel, eluent - 4 L of chloroform / methanol 95 / 5 followed by 30 L of chloroform / methanol / iso-propylamine 95 / 5 / 5, Rf = 0.24) to obtain 13.1 g of the desired spermidine cholesterol carbamate in 64% yield. HPLC (C-18 reversed phase column, linear gradient elution profile - methanol / iso-propanol / water / trifluoroacetic acid 60 / 20 / 20 / 0.1 to methanol / iso-propanol / trifluoroacetic acid 70 / 30 / 0.1 to methanol / iso-propanol / chloroform / trifluoroacetic acid 60 / 20 / 20 / 0.1) analysis of this material showed it to be 99.2% pure with the 7-dehydrocholesterol analog present at a level of 0.8%.

In connection with this example and those that follow, it is noted that all TLC plates were visualized with phosphomolybdic acid.

(B) N^4-Spermine cholesteryl carbamate

Spermine cholesterol carbamate (Figure 1, No. 67) was prepared according to the following procedure which is outlined in Figure 9.

N^1,N^{12}-diCBZ-spermine

Benzylchloroformate (1.76g, 1.5 ml, 10.36 mmol) was dissolved in methylene chloride (5 ml) and placed in a three neck flask under a nitrogen atmosphere. Imidazole (1.4 g, 20.6 mmol) was dissolved in methylene

chloride (20 ml) and placed in an addition funnel. The three neck flask was cooled to 0°C and the imidazole solution was added gradually over 20 min. The mixture was stirred at room temperature for 1 hour and then methylene chloride (25 mL) and citric acid (10%, 25 ml) were added. The layers were separated and the organic fraction was washed with citric acid (10%, 25 ml). The organic component was dried over magnesium sulfate and concentrated in vacuo. The residue was dried under high vacuum for 1 hour at ambient temperature.

To the residue was added dimethylaminopyridine (35 mg), methylene chloride (25 ml) and the mixture was cooled to 0°C, under a nitrogen atmosphere. To an addition funnel was added a solution of spermine (1g, 4.94 mmol) in methylene chloride (25 ml). The spermine solution was added gradually over 15 min. The reaction mixture was stirred overnight at ambient temperature and then concentrated in vacuo. The residue was dissolved in ethyl acetate (80 ml) and washed three times with water (15 ml). The organics were dried over magnesium sulfate, filtered and concentrated in vacuo to give a crude white solid. The material was purified by flash chromatography (65g silica gel, 100:100:10 $CHCl_3$: MeOH: NH_4OH, product Rf.=0.33), to give after drying under high vacuum 1.01g (2.146 mmol, 43 % yield) of product.

N^1,N^{12}-diCBZ- N^4- spermine cholestryl carbamate

Cholesteryl chloroformate (964 mg, 2.15 mmol) was dissolved in chloroform (10 ml) and added dropwise to a cooled (0°C) solution of N^1,N^{12}-diCBZ spermine (1.01g, 2.15 mmol), triethylamine (1 ml) in chloroform (10 ml). The reaction was allowed to warm to room temperature and stirred for 2 hours. To the reaction solution was added water (25 ml) and chloroform (25 ml). The layers were separated and the organic fraction dried over magnesium sulfate. The solution was concentrated in vacuo to give a crude material that was purified by flash chromatography (68g silica gel, MeOH /

$CHCl_3$ 1/4, product Rf. =0.36) to give 1.23 g (1.39 mmol, 65% yield) of product.

final synthesis of N^4-Spermine Cholesteryl Carbamate

N^1,N^{12}-diCBZ-N^4-spermine cholesteryl carbamate (262 mg, 0.300 mmol) was dissolved in 5 ml of acetic acid and 45 mg of 10% Pd on C was added. The solution was purged with nitrogen and stirred under hydrogen at atmospheric pressure. The hydrogenolysis was allowed to proceed for 7 hours. The reaction mixture was filtered and the catalyst was washed with 40 ml of ethyl acetate / acetic acid 9 / 1 and the filtrate will be concentrated *in vacuo* to give a residue. The crude product was dissolved in 35 mL of 1N NaOH and extracted three times with 40 ml of chloroform / methanol 9 / 1. The combined organic fractions were washed with 20 mL of water and dried over Na_2SO_4. The solution was filtered, concentrated *in vacuo* and dried under vacuum to give 125 mg of the desired product in 67% yield.

In connection with the above procedure, it is noted that the hydrogenolysis should be carried out under acidic conditions, in order to minimize the poisoning of the catalyst.

Additional synthesis procedure for N^4-spermine cholesteryl carbamate (No. 67)

(Synthesis of N^1,N^{12} -diCbz-spermine di-HCl salt)

Benzylchloroformate (15 mL, 105 mmol) was dissolved in methylene chloride (335 mL) and placed in a three neck flask under a nitrogen atmosphere. Imidazole (14 g, 206 mmol) was dissolved in methylene chloride (200 mL). The three neck flask was cooled to 0-2 °C using an ice-water bath and the imidazole solution was added gradually over 30 min. The cooling bath was removed and the mixture stirred at room temperature for 1 hour. Methylene chloride (250 mL) and aqueous citric acid (10%, 250 mL) were added to the mixture. The layers were separated and the organic layer was

washed with aqueous citric acid (10%, 250 mL). The organic fraction was dried over magnesium sulfate and concentrated *in vacuo*. The resulting oil was vacuum dried for 2 hours at ambient temperature. To the oil was added dimethylaminopyridine (530 mg, 4.3 mmol) and methylene chloride (250 mL). The mixture was cooled to 0-2 °C and kept under a nitrogen atmosphere. A solution of spermine (10g, 49 mmol) in methylene chloride (250 mL) was added gradually over 15 minutes, maintaining a reaction temperature of 0-2 °C. The reaction mixture was stirred overnight at ambient temperature and then concentrated *in vacuo*. To the resulting material was added 1M hydrochloric acid (67 mL) and methanol (400 mL). The solution was cooled overnight at 4 °C yielding a white precipitate. The precipitate was isolated by vacuum filtration using Whatman #1 filter paper. The N^1,N^{12}-diCbz-spermine di HCl salt (13.38g, 24.7 mmol, 50% yield) thus obtained was dried under vacuum at ambient temperature for 17 hours.

(Synthesis of N^1,N^{12}-diCbz-N^4-spermine cholesteryl carbamate)

N^1,N^{12}-diCbz-spermine di HCl salt (13.38g, 24.7 mmol) was dissolved in a chloroform, methanol and water mixture in the ratio 65:25:4 (940 mL). The solution was stirred at room temperature and cholesteryl chloroformate (11g, 24.5 mmol) was added. The solution was stirred at ambient temperature for 1.5 hours and then diluted with 1M sodium hydroxide solution (165 mL). The organic and aqueous layers were separated and the organic layer containing the product was washed with water (110 mL). The organic fraction was dried over sodium sulfate, concentrated *in vacuo* and vacuum dried. The crude oil was purified by chromatography using silica gel (60Å, 1 Kg) . The silica was packed in 10% MeOH / $CHCl_3$ and the column was eluted with 25% MeOH / $CHCl_3$. Fractions of 900 mL were collected and analyzed by thin layer chromatography. Fractions containing the product (Rf. = 0.5 in 20% MeOH / $CHCl_3$) were combined and concentrated *in vacuo*. The resulting oil was dried

under vacuum for 17 hours to give 8.5g (9.67 mmol, 39% yield) of product.

(Final Synthesis of N^4-spermine cholesteryl carbamate)

N^1,N^{12}-diCbz-N^4-spermine cholesteryl carbamate (8.5g, 9.67 mmol) was dissolved in 200 mL of acetic acid and 1.66 g of 10% Pd on carbon was added. The solution was purged with nitrogen and stirred under hydrogen at atmospheric pressure. The hydrogen was supplied to the reaction flask using a balloon filled with hydrogen gas. The hydrogenolysis was allowed to proceed for 3 hours. The reaction mixture was filtered through Whatman #1 filter paper and the catalyst was washed with 250 mL of 10% acetic acid in ethyl acetate. The filtrate was concentrated *in vacuo* to give a residue, coevaporation with chloroform aids removal of the acetic acid. To the crude product was added 1M sodium hydroxide solution (400 mL) and the solution was extracted three times with 10% MeOH / $CHCl_3$ (700 mL). The combined organic fractions were washed with water (600 mL) and dried over sodium sulfate. The solution was filtered, concentrated *in vacuo* and vacuum dried at ambient temperature for 48 hours. The crude material was purified by chromatography on silica gel (500 g). The column was packed in 40:25 MeOH : $CHCl_3$ and eluted with 40:25 MeOH : $CHCl_3$ and then 40:25:10 MeOH : $CHCl_3$: NH_4OH. The fractions collected were analyzed by thin layer chromatography and the product containing fractions were combined and concentrated *in vacuo* (the evaporation was assisted by the addition of *iso*-propanol in order to azeotrope the residual water). The material was vacuum dried at ambient temperature for 48 hours to give N^4-spermine cholesteryl carbamate (4g, 6.5 mmol, 67% yield).

(C) <u>N^1,N^8-Bis(3-aminopropyl)-N^4-spermidine cholesteryl carbamate</u>

N^1,N^8-Bis(3-aminopropyl)-N^4-spermidine cholesteryl carbamate (Figure 1, No. 75) was prepared according to the following procedure.

N^4-Spermidine cholesteryl carbamate (1.14g, 2.04 mmol) was dissolved in

MeOH (5 mL). Freshly distilled acrylonitrile (0.28 mL, 4.29 mmol) was added and the solution was stirred at room temperature for 18 h. The solvent was concentrated *in vacuo* to give an oil. Vacuum drying was then carried out overnight. The crude product was purified by column chromatography (125 g silica gel, eluent - $CHCl_3$ MeOH 1/9) to give 1.15 g (85 %) of the N^1,N^8-Bis (cyanoethyl) N^4-Spermidine cholesteryl carbamate.

Raney Nickel 50% slurry (1.2 g, Aldrich) was placed in a Parr Bomb with 1M NaOH in 95% EtOH (50 mL). The N^1,N^8-Bis (cyanoethyl) N^4-Spermidine cholesteryl carbamate. was dissolved in EtOH (35 mL) and added to the bomb. The vesicle was evacuated and placed under Argon pressure (80-100 psi), three times and then evacuated and placed under Hydrogen pressure (100 psi), three times. The reaction was stirred under hydrogen pressure (100 psi) at room temperature for 72h. The vesicle was evacuated and placed under argon pressure. The catalyst was removed by filtration. The filtrate was concentrated *in vacuo* . The resulting oil was dissolved in 2:1 CH_2Cl_2: MeOH (100 mL) and washed with H_2O (35 and 25 mL). The organic layer was dried over Na_2SO_4 and filtered. The filtrate was concentrated *in vacuo* and the residue was purified by chromatography on 100 g of silica gel (eluent - $CHCl_3$/MeOH/conc. NH_4OH 40/25/10, sample applied in $CHCl_3$/MeOH 40/25). The purified material was concentrated *in vacuo* with iPrOH (3 X 50 mL) and CH_2Cl_2(3X50 mL) and then vacuum dried to give 986 mg (85%) of N1,N8-Bis(3-aminopropyl)-N^4-spermidine cholesteryl carbamate.

(D) N(N^4-3-aminopropyl-spermidine) cholesteryl carbamate

N(N^4-3-aminopropyl-spermidine) cholesteryl carbamate (Figure 1, No. 78) was prepared as follows:

N^1, N^8-dicarbobenzoxyspermidine (1.0 g, 2.4 mmol) was dissolved in MeOH (10 mL). Freshly distilled acrylonitrile (0.3 mL, 4.5 mmol) was added and the reaction was stirred at room temperature for 18 h. The solvent was

concentrated *in vacuo* to give an oil. The crude product was purified by column chromatography (100 g silica gel, eluent - $CHCl_3$/MeOH 1/19) to give 1.10 g (97 %) of N^4-2-Cyanoethyl-N^1, N^8 - dicarbobenzoxyspermidine.

The N^4-2-Cyanoethyl-N^1, N^8-dicarbobenzoxyspermidine (0.5 g, 1.07 mmol) was dissolved in MeOH (5 mL) and $CoCl_2$ (280 mg, 2.15 mmol, Aldrich) was added. The blue solution was cooled in an ice bath and $NaBH_4$ (405 mg, 10.7 mmol, Aldrich) was added in portions over 15 min. The resulting black solution was stirred at room temperature for 1 h. The black solution turned blue over this period. To the reaction was added CH_2Cl_2/MeOH 2/1 (30 mL). A black ppt formed. To this was added H_2O (20mL) and the mixture was filtered. The resulting layers were separated and the organic layer dried with $MgSO_4$. The drying agent was filtered and the filtrate concentrated *in vacuo* to give an oil. The crude product was purified by column chromatography (50 g silica gel, eluent - $CHCl_3$/MeOH/conc NH_4OH 100/100/5) to give 309 mg (62 %) of the N^4-3-aminopropyl-N^1, N^8 - dicarbobenzoxyspermidine.

To the N^4-3-aminopropyl-N^1, N^8 - dicarbobenzoxyspermidine (300 mg, 0.66 mmol) dissolved in CH_2Cl_2 was added Et_3N under N_2. Cholesteryl chloro formate (326 mg, 0.726 mmol, Aldrich) was dissolved in CH_2Cl_2 and added to the reaction dropwise. The mixture was stirred for 2h at room temperature. After adding CH_2Cl_2 (25 mL) and H_2O (10 mL), the layers were separated. The organic layer was dried with $MgSO_4$ and filtered. The filtrate was concentrated *in vacuo* to give 640 mg of crude product. The residue was purified by chromatography on 80 g of silica gel (eluent - $CHCl_3$ / MeOH 90 /10, sample applied in $CHCl_3$ / MeOH 90/10). The purified material was concentrated *in vacuo* and then vacuum dried to give 329 mg (57%) of N-(N^4-3-aminopropyl-N^1, N^8 - dicarbobenzoxyspermidine) cholesteryl carbamate.

To 10% Pd on carbon (65 mg, Aldrich) was added a solution of N-(N^4-3-

aminopropyl-N^1, N^8 - dicarbobenzoxyspermidine) cholesteryl carbamate (300 mg) in acetic acid (25 mL). The reaction was placed under H_2 and stirred at room temperature overnight. After being placed under N_2, the reaction was filtered. The catalyst was washed with 10 % acetic acid in EtOAc (50 mL). The filtrate was concentrated *in vacuo* to give an oil. The oil was dissolved in 2/1 CH_2Cl_2/MeOH (35 mL) and washed with 1 M NaOH (15 mL). The organic layer was dried with $MgSO_4$ and filtered. The filtrate was concentrated *in vacuo* and vacuum dried to give 196 mg (93%) of N-(N^4-3-aminopropylspermidine) cholesteryl carbamate.

(E) N-[N^1,N^4,N^8-Tris (3-aminopropyl) spermidine] cholesteryl carbamate

N-[N^1,N^4,N^8-Tris (3-aminopropyl) spermidine] cholesteryl carbamate (Figure 1, No. 96) was prepared by reacting N-(N^4-3-aminopropylspermidine) cholesteryl carbamate with acrylonitrile (90% yield) and subsequent reduction of the di adduct with Raney nickel (75 % yield) as described for the preparation of N^1,N^8Bis(3-aminopropyl)-N4-spermidine cholesteryl carbamate.

(F) N^1, N^8-Bis(Arginine carboxamide)-N^4-spermidine cholesteryl carbamate

N^1, N^8-Bis(Arginine carboxamide)-N^4-spermidine cholesteryl carbamate (Figure 5, No. 95) was prepared as follows.

To $N^{(a)}$,$N^{(e)}$,$N^{(e)}$ (alpha, epsilon, epsilon) -tricarbobenzoxyArginine in CH_2Cl_2 (25 mL) was added N-hydroxysuccinimide (100 mg, 0.89 mmol) and dicyclohexylcarbodiimide (240 mg, 0.89 mmol). The mixture was stirred under N_2 at room temperature for 2.5 hours. N^4- Spermidine Cholesteryl Carbamate (250 mg, 0.448 mmol) and Et_3N (0.25 mL, 1.8 mmol) was added and the reaction stirred at room temperature under N_2 for 72 h. The reaction was filtered and the precipitate was washed with CH_2Cl_2 (20 mL). The

filtrate was washed with H_20 (20 mL). The separated organic layer was dried over $MgSO_4$ and filtered. The filtrate was concentrated *in vacuo* and the residue was purified by chromatography on 70 g of silica gel (eluent - $CHCl_3$ / MeOH 95/ 5). The purified material was concentrated *in vacuo* and then vacuum dried to give 533 mg (71%) of N^1, N^8-Bis ($N^{(a)}$,$N^{(e)}$,$N^{(e)}$-tricarbobenzoxyArginine carboxamide)-N^4-spermidine cholesteryl carbamate.

The carbobenzoxy group were removed from N^1, N^8-Bis ($N^{(a)}$,$N^{(e)}$,$N^{(e)}$-tricarbobenzoxyArginine carboxamide)-N^4-spermidine cholesteryl carbamate as described in the preparation of N-(N^4-3-aminopropylspermidine) cholesteryl carbamate. The product, N^1, N^8-Bis(Arginine carboxamide)-N^4-spermidine cholesteryl carbamate was obtained in 27 % yield.

(G) <u>1-(N^4-spermine)-2,3-dilaurylglycerol carbamate</u>

1-(N^4-spermine)-2,3-dilaurylglycerol carbamate (Figure 7, No. 89) was prepared as follows. A solution of 3-benzyloxy-1,2-propanediol (1.00 g, 5.49 mmol) in THF (20 mL) was added to a suspension of sodium hydride (60% w/w in oil, 550 mg, 13.725 mmol) in THF (30 mL) and allowed to reflux overnight under dry nitrogen. A solution of dodecyl methane sulfonate (3.39 g, 12.078 mmol) in THF (20 mL) was added and the reaction was refluxed for another two days. After cooling to room temperature the reaction was filtered through a bed of Celite, rinsing with THF. The filtrate was reduced *in vacuo* to a yellow oil which was redissolved in diethyl ether (100 mL). The ether solution was washed with 0.1 N NaOH (30 mL) and dH2O (2 x 30 mL). The organic layer was dried over magnesium sulfate, filtered and reduced *in vacuo* to a red-brown oil. The crude material was purified by flash column chromatography (300 g silica gel) eluting with 3% ethyl acetate/ hexanes. The desired product was isolated as a pale yellow oil and characterized by ^1H NMR as 3-OBn-1,2-dilaurylglycerol (1.70 g, 60%). 3-OBn-1,2-dilaurylglycerol

(1.70 g, 3.28 mmol) in ethanol (100 mL) was stirred with 10% Pd/C (250 mg, 15 wt%) under a hydrogen atmosphere for 24 hours. The reaction was flushed with nitrogen and filtered through Celite, rinsing with ethanol, to remove the catalyst. The filtrate was reduced *in vacuo* to a solid. The crude material was purified by flash column chromatography (140 g silica gel) eluting with 10% ethyl acetate/ hexanes. The desired product was isolated as a white solid and characterized by ^{1}H NMR as 1,2-dilaurylglycerol (1.23g, 88%).

A 1.93 M solution of phosgene in toluene (0.77 mL, 1.49 mmol) was added to a solution of 1,2-dilaurylglycerol (580 mg, 1.35 mmol) and N,N-diisopropylethylamine (0.26 mL, 1.49 mmol) in methylene chloride (10 mL) and stirred overnight. A solution of N^1,N^{12}-di-CBz-spermine•2HCl (734 mg, 1.35 mmol) in 60: 25: 4 chloroform/ methanol/ water (80 mL) was added. After 3 hours another equivalent of N,N-diisopropylethylamine (0.26 mL, 1.49 mmol) was added. An additional 0.5 equivalents of N,N-diisopropylethylamine (0.13 mL, 0.75 mmol) was added three hours later and the reaction was allowed to stir overnight under nitrogen at ambient temperature. The reaction was washed with 1M NaOH (20 mL) and dH2O (15 mL). The organic layer was separated, dried over magnesium sulfate, filtered and reduced *in vacuo* to a white solid The crude material was purified by flash column chromatography (125 g silica gel) eluting with 90: 10: 0.5 chloroform/ methanol/ ammonium hydroxide. The desired product was isolated as an oil and characterized by ^{1}H NMR as 1-(N^4-(N^1,N^{12}-di-CBz-spermine))-2,3-dilaurylglycerol carbamate (188 mg, 15%).

The 1-(N^4-(N^1,N^{12}-di-CBz-spermine))-2,3-dilaurylglycerol carbamate (188 mg, 0.203 mmol) was dissolved in glacial acetic acid (10 mL) and stirred with 10% Pd/C (45 mg, 24 wt %) under a hydrogen atmosphere for 5 hours. The catalyst was removed by vacuum filtration rinsing with 10% acetic acid/

ethyl acetate (10 mL) The filtrate was reduced to an oil by rotary evaporation. The resulting oil was dissolved in 10% methanol/ chloroform (85 mL) and was washed with 1M NaOH (15 mL) and dH2O (10 mL). The organic layer was separated, dried over magnesium sulfate, filtered and reduced *in vacuo* to an oil. The product was characterized by ^{1}H NMR as 1-(N^4-spermine)-2,3-dilaurylglycerol carbamate (125 mg, 94%).

Other amphiphiles useful in the practice of the invention may be prepared according to procedures that are within the knowledge of those skilled in art.

Examples

The following Examples are representative of the methodology that can be employed to evaluate the effectiveness of the cationic amphiphile/neutral co-lipid compositions of the invention.

Example 1 - Cell Transfection Assay

Separate 3.35 µmole samples of spermidine cholesterol carbamate (amphiphile No. 53) and the neutral lipid dioleoylphosphatidylethanolamine ("DOPE") were each dissolved in chloroform as stock preparations. Following combination of the solutions, a thin film was produced by removing chloroform from the mixture by evaporation under reduced pressure (20 mm Hg). The film was further dried under vacuum (1 mm Hg) for 24 hours. As aforementioned, some of the amphiphiles of the invention participate in transacylation reactions with co-lipids such as DOPE, or are subject to other reactions which may cause decomposition thereof. Accordingly, it is preferred that amphiphile/co-lipid compositions be stored at low temperature, such as -70 degrees C, until use.

To produce a dispersed suspension, the lipid film was then hydrated with sterile deionized water (1 ml) for 10 minutes, and then vortexed for 1

minute (sonication for 10 to 20 seconds in a bath sonicator may also be used, and sonication has proved useful for other amphiphiles such as DC-chol). The resulting suspension was then diluted with 4 ml of water to yield a solution that is 670μM in cationic amphiphile and 670μM in neutral colipid.

Experiments were also performed using spermine cholesterol carbamate (amphiphile No. 67) and other amphiphiles of the invention. With respect to spermine cholesterol carbamate, the optimum molar ratio of amphiphile to DOPE under the conditions tested was determined to be 1:2, not 1:1. Similar optimized ratios may be determined for other amphiphile/neutral co-lipid compositions of the invention.

For preparation of the transfecting solution, DNA encoding for ß-galactosidase (pCMVß, ClonTech., Palo Alto, CA) was dissolved in OptiMEM culture medium (Gibco/ BRL No. 31885-013). The resulting solution had a DNA concentration of 960 μM (assuming an average molecular weight of 330 daltons for nucleotides in the encoding DNA).

The following procedure was used to test a 1:1 molar mixture of the cationic amphiphile spermidine cholesterol carbamate in combination with DOPE. A 165 μl aliquot of spermidine cholesterol carbamate (670 μM) containing also the colipid (at 670 μM) was pipetted into 8 separate wells in a 96-well plate containing OptiMEM (165μl) in each well. The resulting 335 μM solutions were then serially diluted 7 times to generate 8 separate amphiphile-containing solutions having concentrations ranging from 335 μM to 2.63 μM, with each resultant solution having a volume of 165 μl. Thus, 64 solutions were prepared in all, there being 8 wells each of 8 different concentrations of amphiphile/DOPE.

Independently, DNA solutions (165μl, 960μM) were pipetted into 8 wells containing OptiMEM (165 μl), and the resulting 480μM solutions were then serially diluted 7 times to generate 8 separate 165 μl solutions from each

well, with the concentrations of DNA in the wells ranging from 480 μM to 3.75 μM.

The 64 test solutions (cationic amphiphile: neutral lipid) were then combined with the 64 DNA solutions to give separate mixtures in 64 wells, each having a volume of 330μl, with DNA concentrations ranging from 240 μM to 1.875 μM along one axis, and lipid concentrations ranging from 167 μM to 1.32 μM along the other axis. Thus 64 solutions were prepared in all, each having a different amphiphile: DNA ratio and/or concentration. The solutions of DNA and amphiphile were allowed to stand for 15 to 30 minutes in order to allow complex formation.

A CFT-1 cell line (human cystic fibrosis bronchial epithelial cells immortalized with papillomavirus) provided by Dr. James Yankaskas, University of North Carolina, Chapel Hill, was used for the *in vitro* assay. The cells are homozygous for a mutant allele (deletion of phenylalanine at position 508, hereinafter F508) of the gene encoding for cystic fibrosis transmembrane conductance regulator ("CFTR") protein. CFTR is a cAMP-regulated chloride (Cl^-) channel protein. Mutation of the CFTR gene results typically in complete loss (or at least substantial impairment) of Cl^- channel activity across, for example, cell membranes of affected epithelial tissues.

The F508 mutation is the most common mutation associated with cystic fibrosis disease. For a discussion of the properties of the F508 mutation and the genetics of cystic fibrosis disease see, in particular, Cheng et al., Cell, 63, 827-834 (1990). See also Riordan et al., Science, 245, 1066-1073 (1989); published European Patent Application No. 91301819.8 of Gregory et al., bearing publication number 0 446 017 A1; and Gregory et al., Nature, 347, 382-385 (1990).

The cells were cultured in Hams F12 nutrient media (Gibco/ BRL No. 31765-027) supplemented with 2% fetal bovine serum ("FBS", Irvine Scientific,

No. 3000) and 7 additional supplements. Cells were then plated into 96-well tissue culture plates at a density of approximately 7,500 cells/well. Before being used in the assay, cells were allowed to grow for periods of 5-7 days until a confluent pattern had been achieved.

Following the allotted time period, three 96-well plates with CFT-1 cells were aspirated in order to remove the growth medium. The various concentrations of DNA-lipid complex (in 100 µl aliquots) were transferred to each of three 96-well plates bringing the DNA-lipid complexes in contact with the cells. DNA-only/cell and lipid-only/cell control wells were also prepared on one of the three plates.

The 100 µl solutions of DNA-lipid complex were maintained over the cells for 6 hours, after which 50 µl of 30% FBS (in OptiMEM) was added to each well. After a further 20-hour incubation period, an additional 100 µl of 10% FBS in OptiMEM was also added. Following a further 24-hour incubation period, cells were assayed for expression of protein and ß-galactosidase.

For the assays, the resultant medium was removed from the plates and the cells washed with phosphate buffered saline. Lysis buffer (50 µl, 250 mM Tris-HCl, pH 8.0, 0.15% Triton X-100) was then added, and the cells were lysed for 30 minutes. The 96-well plates were carefully vortexed for 10 seconds to dislodge the cells and cell debris, and 5 µl volumes of lysate from each well were transferred to a plate containing 100µl volumes of Coomassie Plus® protein assay reagent (Pierce Company, No. 23236). The protein assay plates were read by a Bio-Rad Model 450 plate-reader containing a 595nm filter, with a protein standard curve included in every assay.

The level of ß-galactosidase activity in each well was measured by adding phosphate buffered saline (50 µl) to the remaining lysates, followed by addition of a buffered solution consisting of chlorophenol red

galactopyranoside (100 µl, 1 mg per ml, Calbiochem No. 220588), 60 mM disodium hydrogen phosphate pH 8.0, 1 mM magnesium sulfate, 10 mM potassium chloride, and 50 mM 2-mercaptoethanol. The chlorophenol red galactopyranoside, following enzymatic (ß-galactosidase) hydrolysis, gave a red color which was detected by a plate-reader containing a 570 nm filter. A ß-galactosidase (Sigma No. G6512) standard curve was included to calibrate every assay.

Following subtraction of background readings, optical data determined by the plate-reader allowed determination of ß-galactosidase activity and protein content. In comparison to the amount of ß-galactosidase expressed by known transfectants, for example, DMRIE (1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide), compounds of the invention are particularly effective in transfecting airway epithelial cells and inducing therein ß-galactosidase expression. Relative to DMRIE:DOPE (1:1), the spermidine cholesterol carbamate: DOPE mixture (also 1:1) demonstrated transfection efficiency improved by a factor of about 5.

Example 2 - Transfection of the Gene Encoding for Human Cystic Fibrosis Transmembrane Conductance Regulator Protein

The ability of the cationic amphiphiles of the invention to transfect cells and to induce therein biochemical corrections was demonstrated with a separate *in vitro* assay. Immortalized human cystic fibrosis airway cells (CFT-1, as above) were used.

In preparation for the assay, the cells were grown on glass coverslips until approximately 60% confluent. The cells were then transfected with a complex of spermidine cholesterol carbamate:DOPE (1:1) and a plasmid(pCMV- CFTR) containing a cDNA that encodes wild type human CFTR. pCMV-CFTR plasmid is a construct containing the encoding sequence

for CFTR and the following regulatory elements, a CMV promoter and enhancer, and an SV40 polyadenylation signal. Additional constructs suitable for the practice of this example include pMT-CFTR, Cheng et al., Cell, 63, 827-834 (1990). The complex used was 10.5 μmolar of spermidine cholesterol carbamate (also of DOPE) and 30 μmolar of pCMV-CFTR based on nucleotide.

48 hours after amphiphile-mediated transfection, cells were tested for cAMP-stimulated Cl^- channel activity using the 6-methoxy-N-(3-sulfopropyl)quinolinium ("SPQ") assay. See S. Cheng et al., Cell, 66, 1027-1036 (1991) for further information concerning assay methodology. In the assay, cAMP-dependent Cl^- channel activity was assessed using "SPQ" (from Molecular Probes, Eugene, Oregon), a halide-sensitive fluorophore. Increases in halide permeability results in a more rapid increase in SPQ fluorescence, and the rate of change (rather than the absolute change in fluorescence) is the important variable in assessing Cl^- permeability. See also Rich et al., Nature, 347, 358-363 (1990) for background information.

Fluorescence of the SPQ molecule in individual cells was measured using an inverted microscope, Nikon, , a digital imaging system from Universal Imaging, and an ICCD camera, Hamamatsu, Inc.. Cells were selected for analysis without prior knowledge of their expected rate-of-change- in-fluorescence characteristics.

In each experiment, up to five microscope fields of between 90 and 100 cells were examined on a given day, and studies under each condition were repeated on at least 3 different days. Since expression of CFTR is heterogenous (i.e. cells do not produce identical amounts of CFTR), the data presented were for the 20% of cells in each field exhibiting the greatest response.

As expected, cells that were mock transfected failed to exhibit any

measurable increase in cAMP-stimulated halide fluorescence. In contrast, cells that had been transfected with the wild type CFTR cDNA displayed a rapid increase in SPQ fluorescence upon stimulation with cAMP agonist, indicating increased permeability to anions. Approximately 60% of the cells assayed exhibited measurable cAMP-stimulated Cl^- channel activity. Accordingly, spermidine cholesterol carbamate, and other cationic amphiphiles of the invention similarly tested, are effective in transferring CFTR-encoding plasmid into immortalized CF airway cells.

Example 3 - CAT Assay

part A

This assay was used to assess the ability of the cationic amphiphiles of the invention to transfect cells in vivo from live specimens. In the assay, the lungs of balb/c mice were instilled intra-nasally (the procedure can also be performed trans-tracheally) with 100 µl of cationic amphiphile:DNA complex, which was allowed to form during a 15-minute period prior to administration according to the following procedure. The amphiphile (premixed with co-lipid, see below) was hydrated in water for 10 minutes, a period sufficient to yield a suspension at twice the final concentration required. This was vortexed for two minutes and aliquoted to provide 55 microliter quantities for each mouse to be instilled. Similarly, DNA encoding the reporter (CAT) gene was diluted with water to a concentration twice the required final concentration, and then aliquoted at 55 microliters for each mouse to be instilled. The lipid was gently combined with the DNA (in a polystyrene tube), and the complex allowed to form for 15 minutes before the mice were instilled therewith.

The plasmid used (pCMVHI-CAT, see Example 4) provides an encoding DNA for chloramphenicol transferase enzyme. Specifics on the amphiphile:DNA complexes are provided below.

Two days following transfection, mice were sacrificed, and the lungs and trachea removed, weighed, and homogenized in a buffer solution (250 mM Tris, pH 7.8, 5mM EDTA). The homogenate was clarified by centrifugation, and the deacetylases therein were inactivated by heat treatment at 70 °C for 10 minutes. Lysate was incubated overnight with acetyl coenzyme A and C^{14} --chloramphenicol. CAT enzyme activity was then visualized by thin layer chromatography ("TLC") following an ethyl acetate extraction. Enzyme activity was quantitated by comparison with a CAT standard curve.

The presence of the enzyme CAT will cause an acetyl group to be transferred from acetylcoenzyme A to C^{14} --chloramphenicol. The acetylated/radiolabeled chloramphenicol migrates faster on a TLC plate and thus its presence can be detected. The amount of CAT that had been necessary to generate the determined amount of acetylated chloramphenicol can then be calculated from standards.

The activity of spermidine cholesterol carbamate (amphiphile No.53) was determined in the CAT assay in relation to the recognized transfection reagents DMRIE and DC-Chol. The enhanced ability of spermidine cholesterol carbamate (amphiphile No. 53) to transfect cells in vivo , was determined to be about 20-fold, or greater, in this assay. In the assay, activity was measured as ng CAT enzyme per 100 mg lung tissue. As a comparison, it is generally observed that DMRIE, a well known transfectant, when prepared as a 1:1 molar mixture with DOPE and then complexed with plasmid DNA (1.7 mM DMRIE, 1.7 mM DOPE, 1.2 mM plasmid DNA measured as nucleotide) gives about 1 to 2 ng activity per 100 mg lung tissue in this assay.

With respect to this comparison, the following conditions are of note. The transfection solution for spermidine cholesterol carbamate contained 6mM DNA measured as concentration of nucleotide, and 1.5 mM of cationic

amphiphile. Following generally the procedure of Example 1, each amphiphile had also been premixed with DOPE, in this case at 1:1 molar ratio. For transfection with DC-chol, the molar ratio of DC-chol to DOPE was 3:2, and the concentrations of cationic amphiphile and of DNA (as nucleotide) were 1.3 mM and 0.9 mM, respectively. For transfection with DMRIE, the molar ratio of DMRIE to DOPE was 1:1 and the concentrations of cationic amphiphile and of DNA were 1.7 mM and 1.2 mM, respectively. These concentrations (and concentration ratios) for each amphiphile, and colipid and DNA, had been determined to be optimal for transfection for that respective amphiphile, and accordingly were used as the basis for the comparison presented herein.

For spermidine cholesterol carbamate (amphiphile No. 53), optimization experiments were also performed to determine preferred concentrations of plasmid for a particular amphiphile concentration, and also to determine preferred concentrations of the same amphiphile in relation to a particular plasmid concentration. Transfection efficiency was optimal at an amphiphile concentration of 1.5 mM (DOPE also being present at 1.5 mM), and about 6 mM (by nucleotide) of plasmid, or about at a ratio of 1:4. It was noted, however, that concentrations of about 0.75 mM of amphiphile, and 3.0 mM of plasmid were less toxic to the target cells.

Intra-nasal transfection with pCMVHI-CAT vector was also performed in mice using spermidine cholesterol carbamate as cationic amphiphile but with cholesterol as co-lipid. In this experiment, the concentrations of spermidine cholesterol carbamate tested were between 1.0 and 1.5 mM (cholesterol being present at a 1:1 molar ratio in each case, with the mixing of amphiphile and co-lipid being performed as above). The DNA concentration (measured as nucleotide concentration) was between 4.0 and 6.0 mM. Transfection efficiency (again measured as ng CAT/100 mg tissue) was less

effective than with DOPE as co-lipid; however, the transfections were substantially more effective than those achieved using DC-Chol/DOPE.

part B

Additional experiments were performed to compare in vivo the transfection efficiency of cationic amphiphiles depicted in Figures 1, 5 and 7. The compounds were administered intra-nasally using between 12 and 15 mice per compound. As in part A above, ng CAT activity was measured per 100 mg of tissue. However, improved vectors (pCF1/CAT and its near equivalent pCF2/CAT) were used. In part resulting from improved vector performance, incubations of lysate with acetyl coenzyme A and C^{14}-chloramphenicol were conducted for only 30 minutes. Construction of pCF1/CAT and pCF2/CAT is described below in Example 4.

The in vivo data were compiled generally as follows. As aforementioned, data was collected from the complete in vivo optimization of amphiphile No. 53. Amphiphile No. 67 was subjected to a similar partial optimization. With respect to all of the other cationic amphiphiles reported on, and taking advantage of numerous structural similarities, optimized compositions for in vivo testing were extrapolated from in vitro results. This facilitated the screening of large numbers of amphiphiles and produced broadly, if not precisely, comparable data. For all amphiphiles other than Nos. 53 and 67, the ratio, for in vivo testing, of amphiphile concentration to DOPE concentration was taken from the in vitro experiments, as was the optimized ratio of amphiphile concentration to DNA concentration (see Example 1). Accordingly, for such amphiphiles the in vivo test concentration was fixed at 1mM, thereby fixing also the co-lipid concentration. [Broadly, the molar ratio of the amphiphile to co-lipid DOPE ranged from 1:2 (for example, spermine cholesterol carbamate, No. 67) through 1:1 (for example, spermidine cholesterol carbamate, No. 53) to about 2:1 (for example,

amphiphile No. 75)]. The concentration of plasmid DNA varied for each amphiphile species tested in order to duplicate the optimized amphiphile/DNA ratio that had been determined in vitro.

part C

That the novel amphiphiles of the invention are an important contribution to the art is immediately seen by comparing their performance - as in vivo transfection enhancers - to that of closely related cationic amphiphiles that lack the novel T-shape. It has been determined that spermidine cholesterol carbamate provides a much greater level of enhancement than N^1-spermidine cholesteryl carbamate which contains the same number of carbon and nitrogen atoms in its cationic alkylamine component but which is linear and not "T-shaped". Following generally the procedures of Example 3, part B, and using respectively 6mM (as nucleotide), 1.5 mM, and 1.5 mM concentrations of DNA, amphiphile and of co-lipid, the transfection enhancement provided by spermidine cholesterol carbamate (amphiphile No.53) , in relation to N^1-spermidine cholesteryl carbamate, was determined to be about 30 fold.

Also following the procedures of Example 3, part B, and using respectively 4mM (as nucleotide), 1mM, and 2 mM concentrations of DNA, amphiphile and co-lipid, the transfection enhancement provided by spermine cholesterol carbamate (amphiphile No. 67) — in relation to N^1-thermospermine cholesteryl carbamate and N^1-spermine cholesteryl carbamate to which spermine cholesterol carbamate is similarly related — is at least about 30 fold.

Example 4- Construction of vectors

As aforementioned, numerous types of biologically active molecules can be transported into cells in therapeutic compositions that comprise one or more of the cationic amphiphiles of the invention. In an important

embodiment of the invention, the biologically active macromolecule is an encoding DNA. There follows a description of novel vectors (plasmids) that are preferred in order to facilitate expression of such encoding DNAs in target cells.

part A — pCMVHI-CAT

pCMVHI-CAT is representative of plasmid constructs useful in the practice of the invention. Although the plasmid is provided in a form carrying a reporter gene (see Example 3), transgenes having therapeutic utility may also be included therein.

The pCMVHI-CAT vector is based on the commercially available vector pCMVß (Clontech). The pCMVß construct has a pUC19 backbone (J. Vieira, et al., Gene , 19, 259-268, 1982) that includes a procaryotic origin of replication derived originally from pBR322. Basic features of the pCMVHI-CAT plasmid (as constructed to include a nucleotide sequence coding for CAT) are as follows. Proceeding clockwise — the human cytomegalovirus immediate early gene promoter and enhancer, a fused tripartite leader from adenovirus and a hybrid intron, a linker sequence, the CAT cDNA, an additional linker sequence, the late SV40 polyadenylation signal, and the pUC origin of replication and backbone that includes the gene for ampicillin resistance.

The human cytomegalovirus immediate early gene promoter and enhancer spans the region from nucleotides 1-639. This corresponds to the region from -522 to +72 relative to the transcriptional start site (+1) and includes almost the entire enhancer region from -524 to -118 as originally defined by Boshart et al, Cell 41:521-530, 1985. The CAAT box is located at nucleotides 487-491 and the TATA box is at nucleotides 522-526 in pCMVHI-CAT. The CAT transcript is predicted to initiate at nucleotide 549, which is the transcriptional start site of the CMV promoter. The tripartite leader-

hybrid intron is composed of a fused tri-partite leader from adenovirus containing a 5' splice donor signal, and a 3' splice acceptor signal derived from an IgG gene. The elements in the intron are as follows: the first leader, the second leader , part of the third leader, the splice donor sequence and intron region from the first leader, and the mouse immunoglobulin gene splice donor sequence. The length of the intron is 230 nucleotides. The CAT coding region comprises nucleotides 1257-1913. The SV40 poly A signal extends from nucleotide 2020 to 2249.

Accordingly, construction of the pCMVHI-CAT plasmid proceeded as follows. The vector pCMVß (Clontech, Palo Alto, CA) was digested with Not I to excise the ß-galactosidase gene. The vector fragment lacking the ß-galactosidase gene was isolated and ligated to form pCMV.

The hybrid intron (Figure 9) was obtained from the plasmid pADß (Clontech). The hybrid intron had been isolated from a 695 base pair XhoI-EcoRI fragment of p91023(B), see Wong et al., Science, 228, 810-815 (1985). The hybrid intron contains the fused tripartite leader from adenovirus, the donor site from the first segment of the tripartite leader, and the acceptor site from an IgG gene, and has a length of 230 bp.

pADß was digested with Pml I and Not I, and the ~500 base-pair (bp) fragment was isolated, and then ligated into the Not I site of pBluescriptII KS(-) (Stratagene, La Jolla, CA) to form pBlueII-HI.

pBlueII-HI was digested with XhoI and NotI to excise the hybrid intron fragment. This fragment was ligated into the XhoI and NotI sites of pCMV, replacing the SV40 intron to form pCMVHI.

The CAT gene was obtained from the Chloramphenicol Acetyltransferase GenBlock (Pharmacia, Piscataway, NJ). This 792 bp Hind III fragment was blunted with the Klenow fragment of DNA Polymerase I, then Not I linkers (New England Biolabs) were ligated to each end. After

digestion with Not I to expose the Not I sticky ends, the fragment was subcloned into the Not I site of pCMV to form pCMV-CAT. pCMV-CAT was digested with Not I to excise the CAT fragment. The CAT fragment was ligated into pCMVHI to form pCMVHI-CAT which is depicted in Figure 8.

part B — pCF1 and pCF2

Although pCMVHI is suitable for therapeutic transfections, further performance enhancements (including increased expression of transgenes) are provided by the pCF1 and pCF2 plasmids. A map of pCF1/CAT is shown in Figure 10, panel A, and a map of pCF2/CAT is shown in panel B.

Briefly, pCF1 contains the enhancer/promoter region from the immediate early gene of cytomegalovirus (CMV). A hybrid intron is located between the promoter and the transgene cDNA. The polyadenylation signal of the bovine growth hormone gene was selected for placement downstream from the transgene. The vector also contains a drug-resistance marker that encodes the aminoglycosidase 3'-phosphotransferase gene (derived from the transposon Tn903, A. Oka et al., Journal of Molecular Biology, 147, 217-226, 1981) thereby conferring resistance to kanamycin. Further details of pCF1 structure are provided directly below, including description of placement therein of a cDNA sequence encoding for cystic fibrosis transmembrane conductance regulator (CFTR) protein.

The pCF1 vector is based on the commercially available vector pCMVß (Clontech). The pCMVß construct has a pUC19 backbone (J. Vieira, et al., Gene, 19, 259-268, 1982) that includes a procaryotic origin of replication derived originally from pBR322.

Basic features of the pCF1-plasmid (as constructed to include a nucleotide sequence coding for CFTR) are as follows. Proceeding clockwise — the human cytomegalovirus immediate early gene promoter and enhancer, a fused tripartite leader from adenovirus and a hybrid intron, a linker

sequence, the CFTR cDNA, an additional linker sequence, the bovine growth hormone polyadenylation signal, pUC origin of replication and backbone, and the kanamycin resistance gene. The pCF1-CFTR plasmid has been completely sequenced on both strands.

The human cytomegalovirus immediate early gene promoter and enhancer spans the region from nucleotides 1-639. This corresponds to the region from -522 to +72 relative to the transcriptional start site (+1) and includes almost the entire enhancer region from -524 to -118 as originally defined by Boshart et al., Cell, 41, 521-530 (1985). The CAAT box is located at nucleotides 486-490 and the TATA box is at nucleotides 521-525 in pCF1-CFTR. The CFTR transcript is predicted to initiate at nucleotide 548, which is the transcriptional start site of the CMV promoter.

The hybrid intron is composed of a fused tri-partite leader from adenovirus containing a 5' splice donor signal, and a 3' splice acceptor signal derived from an IgG gene. The elements in the intron are as follows: the first leader (nucleotides 705-745), the second leader (nucleotides 746-816), the third leader (partial, nucleotides 817-877), the splice donor sequence and intron region from the first leader (nucleotides 878-1042), and the mouse immunoglobulin gene splice donor sequence (nucleotides 1043-1138). The donor site (G | GT) is at nucleotides 887-888, the acceptor site (AG | G) is at nucleotides 1128-1129 , and the length of the intron is 230 nucleotides. The CFTR coding region comprises nucleotides 1183-5622.

Within the CFTR-encoding cDNA of pCF1-CFTR, there are two differences from the originally-published predicted cDNA sequence (J. Riordan et al., Science, 245, 1066-1073, 1989); (1) an A to C change at position 1990 of the CFTR cDNA which corrects an error in the original published sequence, and (2) a T to C change introduced at position 936. The change at position 936 was introduced by site-directed mutagenesis and is silent but

greatly increases the stability of the cDNA when propagated in bacterial plasmids (R. J. Gregory et al. et al., Nature , 347, 382-386, 1990). The 3' untranslated region of the predicted CFTR transcript comprises 51 nucleotides of the 3' untranslated region of the CFTR cDNA, 21 nucleotides of linker sequence and 114 nucleotides of the BGH poly A signal.

The BGH poly A signal contains 90 nucleotides of flanking sequence 5' to the conserved AAUAAA and 129 nucleotides of flanking sequence 3' to the AAUAAA motif. The primary CFTR transcript is predicted to be cleaved downstream of the BGH polyadenylation signal at nucleotide 5808. There is a deletion in pCF1-CFTR at position +46 relative to the cleavage site, but the deletion is not predicted to effect either polyadenylation efficiency or cleavage site accuracy, based on the studies of E.C. Goodwin et al., J. Biol. Chem., 267, 16330-16334 (1992). After the addition of a poly A tail, the size of the resulting transcript is approximately 5.1 kb.

pCF2 plasmid, Figure 10(B), contains a second CMV enhancer, in tandem with the first. Enhanced expression of transgenes from pCF1 or pCF2 results from the combination of a strong promoter, the presence of a highly efficient polyadenylation signal, a leader sequence that enhances translation, and an intron to increase message stability.

Example 5- Correction of Chloride Ion Transport Defect in Nasal Polyp Epithelial Cells of a Cystic Fibrosis Patient by Cationic Amphiphile-Mediated Gene Transfer

Primary (non-immortalized) nasal polyp cells from an adult male cystic fibrosis patient (homozygous for the F508mutation) were grown on collagen- coated permeable filter supports (Millicells) to form a polarized and confluent epithelial monolayer. Once the monolayer was electrically tight (about 5 to 7 days post seeding, and as indicated by the development of resistance across the cell sheet), the apical surface can be exposed to

formulations of cationic amphiphile: DNA complex.

In this case, the amphiphile (spermidine cholesterol carbamate) was provided as a 1:1 (by mole) mixture with DOPE, and this mixture was then complexed with pCMV-CFTR plasmid vector (a construct encoding wild type human cystic fibrosis transmembrane conductance regulator protein, see above). Concentrations in the final mixture were 42 μmolar of spermidine cholesterol carbamate(and also of DOPE) and 60 μmolar (based on molarity in nucleotides) of the plasmid expression vector.

Expression of CFTR was determined by measuring cAMP-stimulated transepithelial chloride secretion in a modified Ussing chamber, Zabner et al., Nature Genetics ,6, 75-83 (1984). The mucosal side of the epithelium was bathed in Ringer's bicarbonate solution bubbled with 95% O_2 and 5% CO_2. The composition of the submucosal solution was similar to the mucosal solution with the exception that sodium gluconate replaced sodium chloride. Transepithelial voltage was clamped to 0 mV and short circuit current was recorded. Amiloride (10 μM) was applied into the apical bath, followed by the mucosal addition of forskolin and IBMX (at 100 μM each). 5-nitro-2-(3-phenylpropylamino) benzoic acid ("NPPB") , an inhibitor of CFTR chloride channels, was then added to the mucosal solution at 10 to 30 μM.

Chloride secretion (i.e. movement of chloride from the epithelial cells to the mucosal solution) was measured as upward deflection. The same plasmid vector, but containing a reporter gene, was used as a negative control. A cAMP-stimulated current (0.5 to 2.5 μampere/cm^2) was observed in monolayers transfected with wild type CFTR gene. Current was not detected with the pCMV-ß-galactosidase control.

Example 6- Correction of Chloride Ion Transport Defect in Airway Epithelial Cells of a Cystic Fibrosis Patient by Cationic Amphiphile-Mediated Gene Transfer

A recommended procedure for formulating and using the pharmaceutical compositions of the invention to treat cystic fibrosis in human patients is as follows.

Following generally the procedures described in Example 1, a thin film (evaporated from chloroform) is produced wherein spermine cholesterol carbamate (amphiphile No. 67) and DOPE are present in the molar ratio of 1: 2. The amphiphile-containing film is rehydated in water-for -injection with gentle vortexing to a resultant amphiphile concentration of about 3mM. However, in order to increase the amount of amphiphile/DNA complex that may be stably delivered by aerosol as a homogeneous phase (for example, using a Puritan Bennett Raindrop nebulizer from Lenexa Medical Division, Lenexa, KS, or the PARI LC Jet™ nebulizer from PARI Respiratory Equipment, Inc., Richmond, VA), it may be advantageous to prepare the amphiphile-containing film to include also one or more further ingredients that act to stablize the final amphiphile/DNA composition. Accordingly, it is presently preferred to prepare the amphiphile-containing film as a 1: 2 : 0.05 molar mixture of amphiphile No. 67, DOPE, and PEG(5000)-DMPE. [A suitable source of PEG-DMPE, polyethylene glycol 5000 - dimyristoylphoshatidyl ethanolamine, is Catalog No. 880210 from Avanti Polar Lipids, Alabaster, AL]. Additional fatty acid species of PEG-PE may be used in replacement therefor.

Without being limited as to theory, PEG(5000)-DMPE is believed to stablize the therapeutric compositions by preventing further agrregation of formed amphiphile/DNA complexes. Additionally it is noted that PEG(2000)-DMPE was found to be less effective in the practice of the invention.

pCF1-CFTR plasmid (containing an encoding sequence for human cystic fibrosis transmembrane conductance regulator, see Example 4) is

provided in water-for-injection at a concentration, measured as nucleotide, of 4 mM. Complexing of the plasmid and amphiphile is then allowed to proceed by gentle contacting of the two solutions for a period of 10 minutes.

It is presently preferred to deliver aerosolized DNA to the lung at a concentration thereof of between about 2 and about 12 mM (as nucleotide). A sample of about 10 to about 40 ml is generally sufficient for one aerosol administration to the lung of an adult patient who is homozygous for the F508 mutation in the CFTR-encoding gene.

It is expected that this procedure (using a freshly prepared sample of amphiphile/DNA) will need to be repeated at time intervals of about two weeks, but depending considerably upon the response of the patient, duration of expression from the transfected DNA, and the appearance of any potential adverse effects such as inflammation, all of which can be determined for each individual patient and taken into account by the patient's physicians.

One important advantage of the cationic amphiphiles of the present invention is that they are substantially more effective — in vivo — than other presently available amphiphiles, and thus may be used at substantially lower concentrations than known cationic amphiphiles. There results the opportunity to substantially minimize side effects (such as amphiphile toxicity, inflammatory response) that would otherwise affect adversely the success of the gene therapy. A further particular advantage associated with use of many of the amphiphiles of the invention should again be mentioned. Many of the amphiphiles of the invention were designed so that the metabolism thereof would rapidly proceed toward relatively harmless biologically-compatible components. In this regard, highly active amphiphiles 53, 67, and 75 are of particular note.

Alternate Procedure to Prepare an Amphiphile/Co-lipid Composition

In order to formulate material that is suitable for clinical

administration, it may be preferable to avoid use of chloroform when the cationic amphiphile and the co-lipid are prepared together. An alternate method to produce such compositions is suggested using formulation of amphiphile 67 (N^4- spermine cholestryl carbamate, Figure 1A) as the example.

The cationic amphiphile, the neutral co-lipid DOPE, and PEG(5000)-DMPE are weighed into vials, and each is dissolved in t-butanol:water 9:1 with vortexing, followed by transfer to a single volumetric flask. An appropriate amount of each lipid is selected to obtain a molar ratio of cationic amphiphile to DOPE to DMPE-PEG of 1 : 2 : 0.05. The resultant solution is vortexed, and further diluted as needed with t-butanol:water 9:1, to obtain the desired concentration. The solution is then filtered using a sterile filter (0.2 micron, nylon).

One mL of the resultant filtered 1 : 2 : 0.05 solution is then pipetted into individual vials. The vials are partially stoppered with 2-leg butyl stoppers and placed on a tray for lyophilization. The t-butanol:water 9:1 solution is removed by freeze drying over 2 to 4 days at a temperature of approximately -5°C. The lyophilizer is then backfilled with argon that is passed through a sterile 0.2 micron filter. The stoppers are then fully inserted into the vials, and the vials are then crimped shut with an aluminum crimp-top. The vials are then maintained at -70°C until use .

Example 7 - In Vivo Evaluation of Dilinoleoyl-sn-Glycero-3-Phospho-ethanolamine-(18:2) Co-lipid.

Figure 11 shows the result on an optimization experiment for in vivo expression in the lungs of balb/c mice (following generally the CAT assay procedure of Example 3) using a reporter gene with spermine cholesterol carbamate (No. 67) as cationic amphiphile. Results are reported in relation to expression (set at 100%) determined for a 1:2 molar ratio mixture of No. 67

amphiphile: DOPE. (DOPE is designated as 18:1c (cis) in the figure).

Comparison was made for a particular composition containing the trans isomer of DOPE (dielaidoyl [18:1 9 trans]-sn-glycero-3-phosphoethanolamine), and also various amphiphile/co-lipid mixtures wherein the neutral co-lipid was dilinoleoyl-sn- glycero -3-phosphoethanolamine (18:2 cis, Catalog No. 850755 from Avanti Polar Lipids, Alabaster, Al).

It was determined that at the directly comparable 1:2 (cationic amphiphile/neutral co-lipid) molar ratio, an approximate 7-fold increase in expression was achieved using the 18:2 co-lipid. An improvement of this magnitude is of considerable importance for therapeutic compositions useful for gene therapy.

Example 8

Figure 12 demonstrates expression of a reporter gene in Cos-1 cells using DC-chol as cationic amphiphile, with various combinations of neutral co-lipids.

Measurement of uptake is made difficult by the need to distinguish DNA that is merely adhering to cells from that which actually enters the cells (see below).

Meaurement of true uptake and resultant expression, based on light units detected, showed that dilinolenoyl-sn-glycero-3-phosphoethanolamine (18:3, Avanti Polar Lipids, catalog no. 850795 was more effective than DOPE alone as co-lipid. It was also demonstrated that although 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (Avanti Polar Lipids, Catalog No. 850757) was relatively ineffective at facilitating expression if used as the only co-lipid, when used in combination with dilinolenoyl-sn-glycero-3-phosphoethanolamine, very substantial expression was achieved.

Example 9

Figure 13 also presents reporter gene expression data, measured as ng CAT detected(see Example 3). Spermine cholesterol carbamate (No. 67) was used as cationic amphiphile, and various species of neutral co-lipid were used.

The 1-palmitoyl and 1-oleoyl species of 1-acyl-2-hydroxy-Sn-glycero-3-phosphoethanolamine (Avanti Polar Lipids, Catalog Nos. 856705 and 846725) were relatively ineffective when used, separately, as the only co-lipid. However, when used in combination with dilinoleoyl-sn-glycero-3-phosphoethanolamine, (18:2, Avanti, catalog 850755) substantial expression was achieved. Activity of trans-unsaturated species was also demonstrated.

With respect to the results reported above in Examples 8 and 9, the following experimental prcoedures were generally applicable.

Cells and culture

Cos-1 cells were cultured in DMEM (high glucose) supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were seeded at 2.6×10^5 cells/well onto 24-well plates 24 h prior to transfection. Cells were about 70% confluent at the time of transfection.

Reagents

Plasmids--To assess expression in mammalian cells a plasmid containing either (1) the luciferase cDNA driven by the RSV promoter pRSVLuc, (2) a plasmid containing the cDNA for B-Galactosidasé driven by the CMV promoter pCMVBGal, or (3) a plasmid containing the cDNA for B-Galactosidase driven by the T7 promoter, pTMBGal was used. Plasmid DNA was purified on Qiagen columns (Quiagen, Inc., Chatsworth, CA). The ^{32}P-labeled pRSVLuc was prepared by labeling 50 ng plasmid DNA with 50 µCi of ^{32}P-dCTP (ICN Radiochemicals, Irvine, CA) according to a protocol provided with the oligolabelling kit (Pharmacia Biotech, Piscataway, NJ). The

labeled plasmid was then separated from unincorporated ^{32}P-dCTP with a Sephadex G-50 Nick Column (Pharmacia Biotech) eluting with 400 µl TE buffer (10mM Tris, 1 mM EDTA, pH = 7).

Vaccinia Viris--To evaluate expression in the cytoplasm without the need for plasmid DNA to enter the nucleus, we used the vaccinia virus/T7 hybrid expression system. This system uses infection with a recombinant vaccinia virus that expresses the T7 RNA polymerase (vTF7-3) and transfection with a plasmid in which the T7 promoter drives B-Galactosidase expression (pTM-BGal).

Lipids-- Lipid stock solutions (in $CHCl_3$) were kept at -20 C under argon in the dark. The cationic component and the PE analog were mixed at 0° C in chloroform the day prior to the experiment. The chloroform solution was subsequently dried down to a lipid film under a stream of argon and stored at -20° C until use. At the time of transfection, the lipid film was allowed to come to room temperature, hydrated with sterile water for injection (Abbott Laboratories, Chicago, IL) at 1µg total lipid/µL water. The lipid film was resuspended by rapidly vortexing for 1 minute with a Fisher Vortex Genie 2 (Scientific Instruments, Bohemia, NY).

Transfections

Preparation of Complexes and Transfection--Plasmid DNA and resuspended lipid were both prediluted in Eagles's MEM at 15-20° C, mixed by inversion, and allowed to incubate at room temperature for 10-15 min before applying to cells. The transfection mixture was left on the cells for 6 h at which time half the cultures were harvested for the measurement of cell-associated radiolabeled plasmid while the media was replaced on the other cultures. These cultures were harvested for evaluation of expression data at 24 h. The assay conditions are as stated below.

Assays

Luciferase Activity-- Luciferase activity was asayed using a kit purchased from Promega (Madison, WI) and a luminometer (Monolight 2010, Analytical Luminescence Laboratory, San Diego, CA). Cells were removed from dishes by incubation with lysis buffer (25mM Tris-phosphate, pH 7.8: 2mM DTT, 2mM 1,2 diaminocyclohexane-N,N,N',N'-tetraacetic acid; 10% glycerol; and 1% Triton X-100) for 15 min followed by scraping. A 4µl aliquot from each well was used for each luciferase assay. Data for luciferase activity represent total values from all cells in one well. In each experiment, triplicate cultures were used for each condition.

B-Galactosidase Activity--- ß-galactosidase activity was assayed using a Galacto-Lite kit purchased from Tropix, Inc. (Bedford, MA) and a luminometer (Monolight 2010, Analytical Luminescence Laboratory, San Diego, CA). Cells were removed from dishes by incubation with 120 µ lysis buffer (100 mM potassium phosphate pH 7.8, 0.2% Triton X-100, and 1 mM dithiothreitol) for 15 minutes followed by scraping. A 4µl aliquot from each well was used for the Galacto-Lite assay. Data for B-Galactosidase activity represent total values from all cells in one well. In each experiment, triplicate cultures were used for each condition.

Protein Assesment --A 3 µl aliquot of the above cell lysate was diluted to 500 µl with water. A 500 µl portion of Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA) was added to this and the absorbance at 595 nm was measured.

Cell-associated DNA Assesment--Cells were harvested after six hours of exposure to the ^{32}P-DNA spiked cationic lipid:DNA complex (240 µg of DNA was spiked with 12.5 ng of ^{32}P-labeled DNA, preparation described above) for the assessment of cell-associated radiolabeled DNA. The wells were washed four times with PBS pH 7.4, then lysis buffer (as above) was

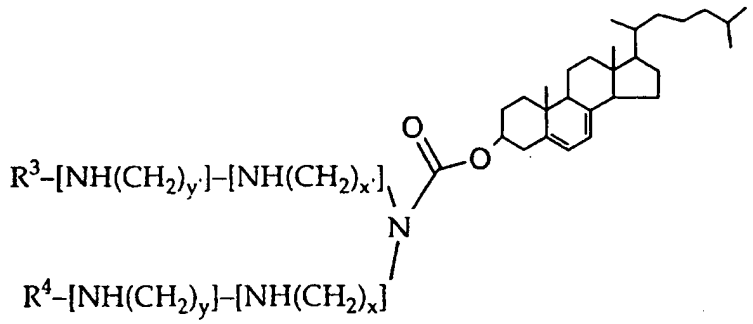
applied, the wells were scraped, and the cell lysate counted in a Rackbeta Model 1209 Liquid Scintillation Counter (LKB Wallac, Gaithersburg, MD). Several washing procedures were tried to determine an optimum procedure for removing non-internalized cationic lipid:DNA complex from the cells. None were found to be more effective than the multiple PBS washing. In addition, incubating the cells with phospholipase D and Dnase was also performed. Neither enzyme increased the radioactivity in the wash over that of PBS. It is possible that the cationic lipid:DNA complex is partially internalized and partially partitioned into the membrane such that it is not susceptible to removal by a washing procedure. It has been shown that DNA is protected from DNase by complexing with cationic lipid .

X-Gal staining -- 24 h after transfection, cells were fixed with 1.8% formaldehyde and 2% gluteraldehyde and then incubated for 16 hr. at 37° C with 0.313 µl of 40 mg/ml X-Gal (5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside) in DMSO dissolved in 12.5 ml of PBS (pH 7.8). Blue staining of nuclei was evaluated by light microscopy. Results are expressed as a precentage; at least 1000 cells were counted in each experiment.

Claims

1. A composition comprising a solid or liquid dispersion of:

(A) a cationic amphiphile according to the formula



wherein:

each of x, x', y, and y' is a whole number other than 0 or 1, except that, optionally,

the entire term $[NH(CH_2)_{y'}]$ can be absent;

R^3 and R^4 are hydrogen;

the total number of nitrogen and carbon atoms in an

R^3— $[NH(CH_2)_{y'}]$— $[NH(CH_2)_{x'}]$ group, or in an

R^4— $[NH(CH_2)_y]$— $[NH(CH_2)_x]$ group, is less that about 30; and

and wherein, optionally, the double bond at C5, and/or the double bond at C7,

in the steroid ring is hydrogenated, and

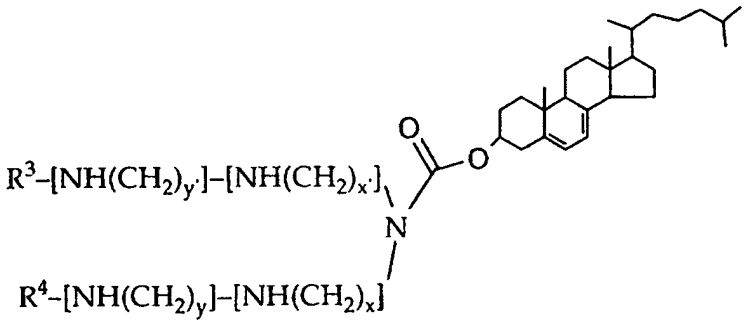
(B) a 1-acyl-2-acyl-sn-glycero-3-phosphoethanolamine having at least two positions of unsaturation in each acyl (fatty acid) chain thereof wherein said fatty acid chains have, individually, from about 12 to about 22 carbon atoms, and are the same or different.

2. A composition according to Claim 1 wherein each of the double bonds in the acyl chains of the 1-acyl-2-acyl-sn-glycero-3-phosphoethanolamine thereof is, independently, either cis or trans.

3. A composition according to Claim 1 wherein the 1-acyl and 2-acyl chains of the 1-acyl-2-acyl-sn-glycero-3-phosphoethanolamine thereof are independetly selected from the group consisting of linoleoyl (18:2), linolenoyl (18:3), arachidonoyl (20:4), and docosahexaenoyl(22:6).

4. A composition comprising a solid or liquid dispersion of:

(A) a cationic amphiphile according to the formula



wherein:

each of x, x', y, and y' is a whole number other than 0 or 1, except that, optionally,

the entire term [NH(CH2)y'] can be absent;

R^3 and R^4 are hydrogen;

the total number of nitrogen and carbon atoms in an

R^3— [NH(CH2)y']— [NH(CH2)x'] group, or in an

R^4— [NH(CH2)y]— [NH(CH2)x] group, is less that about 30; and

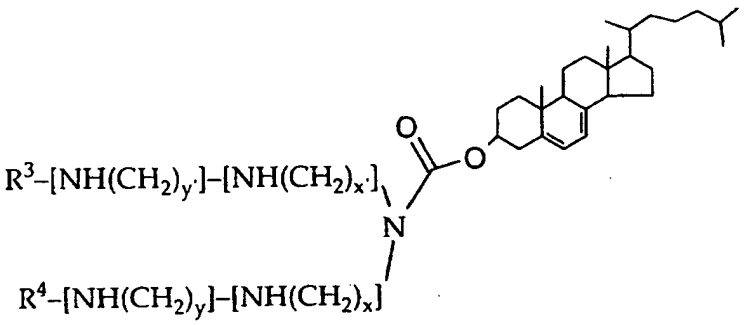
and wherein, optionally, the double bond at C5, and/or the double bond at C7,

in the steroid ring is hydrogenated, and.

(B) a 1-acyl-2-acyl-sn-glycero-3-phosphoethanolamine having at least one position of unsaturation in each acyl (fatty acid) chain thereof wherein said fatty acid chains have, individually, from about 12 to about 22 carbon atoms, and are the same or different, and at least one fatty acid chain therein is selected from the group consisting of elaidoyl (18:1 9 trans), and palmitoleoyl (16:1).

5. A composition comprising a solid or liquid dispersion of:

(A) a cationic amphiphile according to the formula



wherein:

each of x, x', y, and y' is a whole number other than 0 or 1, except that, optionally,

the entire term $[NH(CH_2)_{y'}]$ can be absent;

R^3 and R^4 are hydrogen;

the total number of nitrogen and carbon atoms in an

R^3— $[NH(CH_2)_{y'}]$— $[NH(CH_2)_{x'}]$ group, or in an

R^4— $[NH(CH_2)_y]$— $[NH(CH_2)_x]$ group, is less that about 30; and

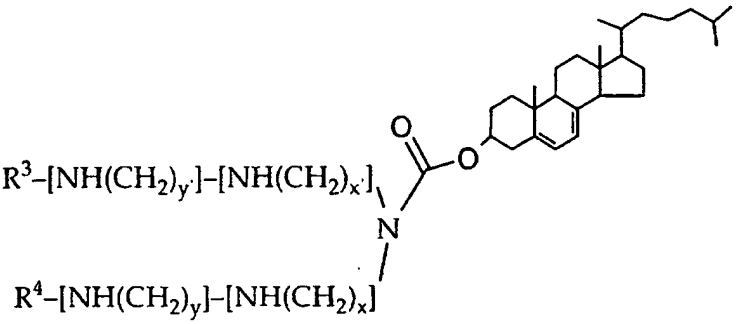
and wherein, optionally, the double bond at C5, and/or the double bond at C7,

in the steroid ring is hydrogenated, and

(B) diphytanoyl {16:0 [$(CH_3)_4$]} -sn-glycero-3-phosphoethanolamine.

6. A composition comprising a solid or liquid dispersion of:

(A) a cationic amphiphile according to the formula



wherein:

each of x, x', y, and y' is a whole number other than 0 or 1, except that,

optionally,

the entire term $[NH(CH_2)_{y'}]$ can be absent;

R^3 and R^4 are hydrogen;

the total number of nitrogen and carbon atoms in an

R^3— $[NH(CH_2)_{y'}]$— $[NH(CH_2)_{x'}]$ group, or in an

R^4— $[NH(CH_2)_y]$— $[NH(CH_2)_x]$ group, is less that about 30; and

and wherein, optionally, the double bond at C5, and/or the double bond at C7,

in the steroid ring is hydrogenated, and.

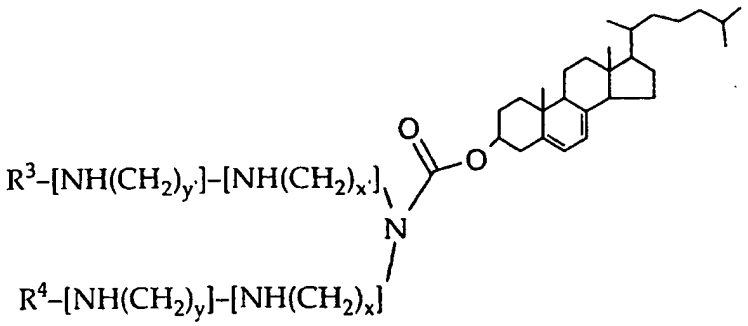
(B) a 1-acyl-2-hydroxy-Sn-glycero-3-phosphoethanolamine wherein the fatty acid chain thereof is from about 12 to about 22 carbon atoms.

7. A composition according to Claim 6 wherein the acyl chain in the 1-acyl-2-hydroxy-Sn-glycero-3-phosphoethanolamine thereof is selected from the

group consisting of palmitoyl (16:0), stearoyl (18:0), and oleoyl (18:1).

8. A composition comprising a solid or liquid dispersion of:

(A) a cationic amphiphile according to the formula



wherein:

each of x, x', y, and y' is a whole number other than 0 or 1, except that, optionally,

the entire term $[NH(CH_2)_{y'}]$ can be absent;

R^3 and R^4 are hydrogen;

the total number of nitrogen and carbon atoms in an

R^3— $[NH(CH_2)_{y'}]$— $[NH(CH_2)_{x'}]$ group, or in an

R^4— $[NH(CH_2)_y]$— $[NH(CH_2)_x]$ group, is less that about 30; and

and wherein, optionally, the double bond at C5, and/or the double bond at C7,

in the steroid ring is hydrogenated;

(B) a 1-acyl-2-acyl-sn-glycero-3-phosphoethanolamine having at least one position of unsaturation in each acyl (fatty acid) chain thereof wherein said fatty acid chains have, individually, from about 12 to about 22 carbon atoms, and are the same or different; and

(C) a 1-acyl-2-hydroxy-Sn-glycero-3-phosphoethanolamine wherein the fatty acid chain thereof is from about 12 to about 22 carbon atoms.

9. A composition according to Claim 8 wherein component (B) is selected from the group consisting of dipalmitoleoyl-sn-glycero-3-phosphoethanolamine and dilinoleoyl-sn-glycero-3-phosphoethanolamine, and component (C) is selected from the group consisting of 1-palmitoyl-2-hydroxy-Sn-glycero-3-phosphoethanolamine and 1-oleoyl-2-hydroxy-Sn-glycero-3-phosphoethanolamine.

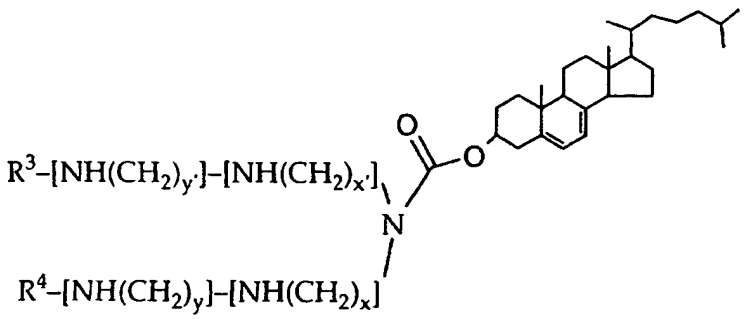
Claim 10. A composition that comprises

(A) a composition according to Claim 1; and

(B) a nucleic acid molecule selected from the group consisting of ribosomal RNA; an antisense polynucleotide whether of RNA or DNA; a ribozyme; and a polynucleotide of genomic DNA, cDNA, or mRNA that encodes for a therapeutically useful protein.

Claim 11. A composition comprising a solid or liquid dispersion of:

(A) a cationic amphiphile according to the formula



wherein:

each of x, x', y, and y' is a whole number other than 0 or 1, except that, optionally,

the entire term $[NH(CH_2)_{y'}]$ can be absent;

R^3 and R^4 are hydrogen;

the total number of nitrogen and carbon atoms in an

R^3— [$NH(CH_2)_{y'}$]— [$NH(CH_2)_{x'}$] group, or in an

R^4— [$NH(CH_2)_y$]— [$NH(CH_2)_x$] group, is less that about 30; and

and wherein, optionally, the double bond at C5, and/or the double bond at C7,

in the steroid ring is hydrogenated;

(B) a first co-lipid according to the formula 1-acyl-2-acyl-sn-glycero-3-phosphoethanolamine having at least two positions of unsaturation in each acyl (fatty acid) chain thereof wherein said acyl chains have, individually, from about 16 to about 18 carbon atoms, and are the same or different; and

(C) a second co-lipid selected from the group consisting of:

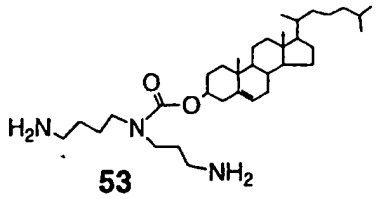
(1) a 1-acyl-2-acyl-sn-glycero-3-phosphoethanolamine wherein each acyl chain has from about 16 to about 18 carbon atoms, and wherein said 1-acyl chain is fully saturated, and said 2-acyl chain has at least one position of unsaturation; and

(2) a 1-acyl-2-hydroxy-Sn-glycero-3-phosphoethanolamine wherein said acyl group thereof has from about 16 to about 18 carbon atoms.

Claim 12. A composition according to Claim 11 wherein:

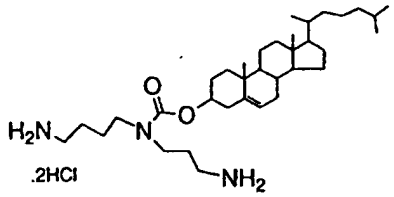
said first co-lipid (B) thereof is selected from the group consisting of dilinoleoyl-sn-glycero-3-phosphoethanolamine and dilinolenoyl-sn-glycero-3-phosphoethanolamine; and said second co-lipid (C) that is selected from the groups consisiting of: for (C1) 1-palmitoyl -2-oleoyl-sn-glycero-3-phosphoethanolamine, 1-palmitoyl -2-linoleoyl-sn-glycero-3-phosphoethanolamine, and 1-palmitoyl -2-linolenoyl-sn-glycero-3-phosphoethanolamine; and for (C2) 1-palmitoyl-2-hydroxy-Sn-glycero-3-phosphoethanolamine, 1-stearoyl-2-hydroxy-Sn-glycero-3-

phosphoethanolamine, 1-oleoyl-2-hydroxy-Sn-glycero-3-phosphoethanolamine, and 1-linoleoyl-2-hydroxy-Sn-glycero-3-phosphoethanolamine.



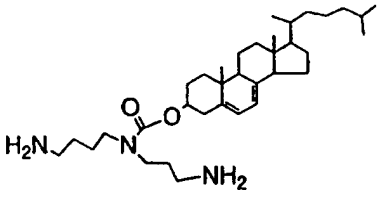
53

N^4-spermidine cholesteryl carbamate



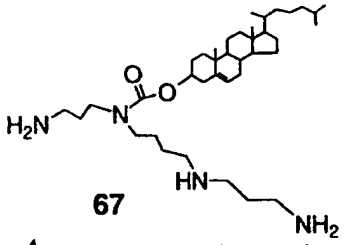
61

N^4-spermidine cholesteryl carbamate di HCl salt



65

N^4-spermidine-7 dehydro cholesteryl carbamate



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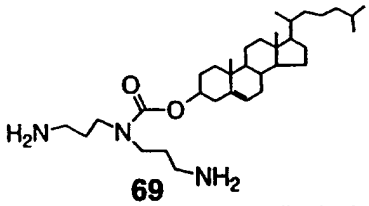
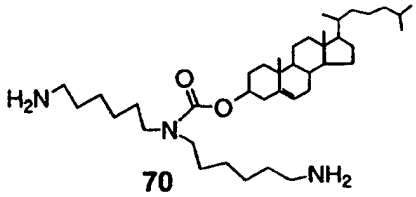
N^4-spermine cholesteryl carbamate



69

N,N bis (3-aminopropyl) cholesteryl carbamate



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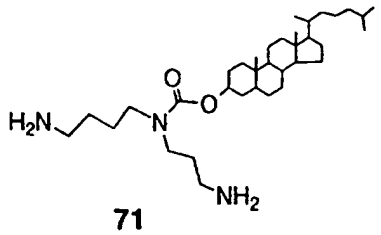
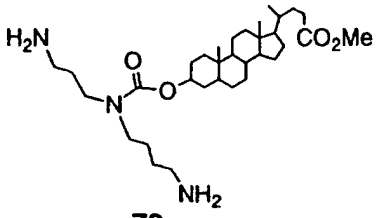
N,N bis (6-aminohexyl) cholesteryl carbamate



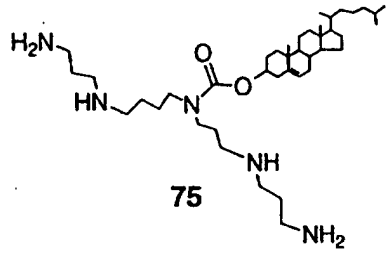
71

N^4-spermidine dihydrocholesteryl carbamate



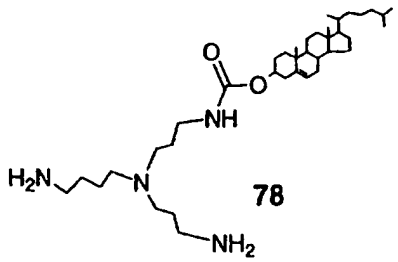
72

N^4-spermidine lithocholic carbamate methyl ester



75

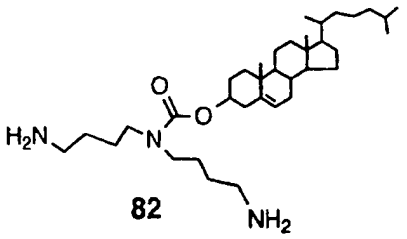
N^1,N^8-Bis (3-aminopropyl)- N^4 - spermidine cholesteryl carbamate



78

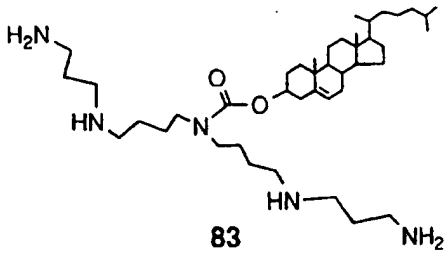
N(N^4-3-aminopropylspermidine) cholesteryl carbamate

FIG. 1 A



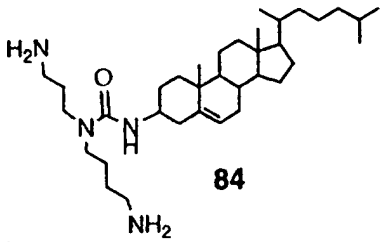
82

N,N-Bis(4-aminobutyl) cholesteryl carbamate



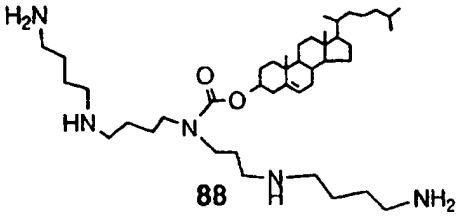
83

N,N-Bis(N'-3-aminopropyl-N''-4-aminobutyl) cholesteryl carbamate



84

N^4-spermidine cholesteryl urea



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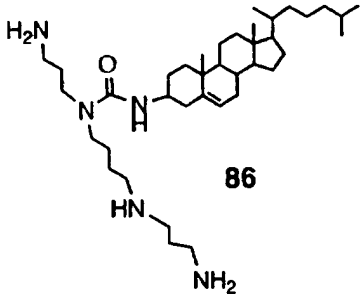
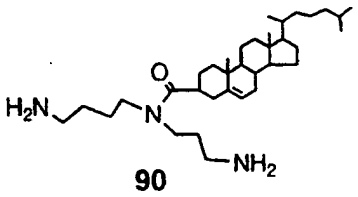


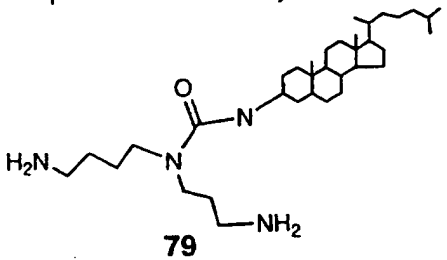
86

N^4-spermine cholesteryl urea



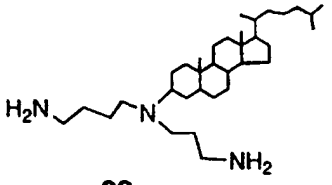
90

N^4 Spermidine cholesteryl carboxamide



79

N^4-spermidine dihydro cholesteryl urea



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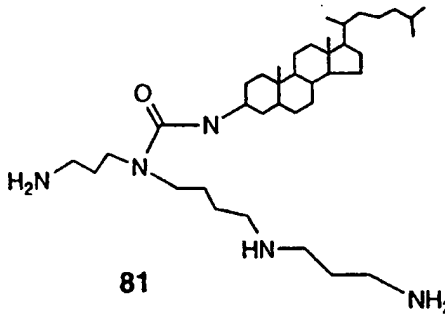
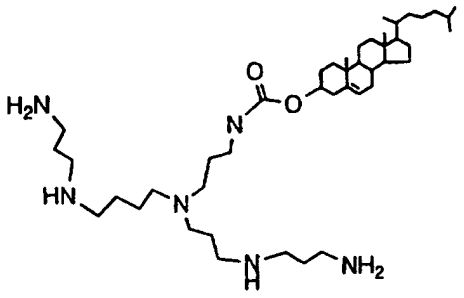


81

N^4-spermine dihydro cholesteryl urea



96

N-[N^1,N^4,N^8-Tris (3-aminopropyl) spermidine] cholesteryl carbamate

FIG. 1B

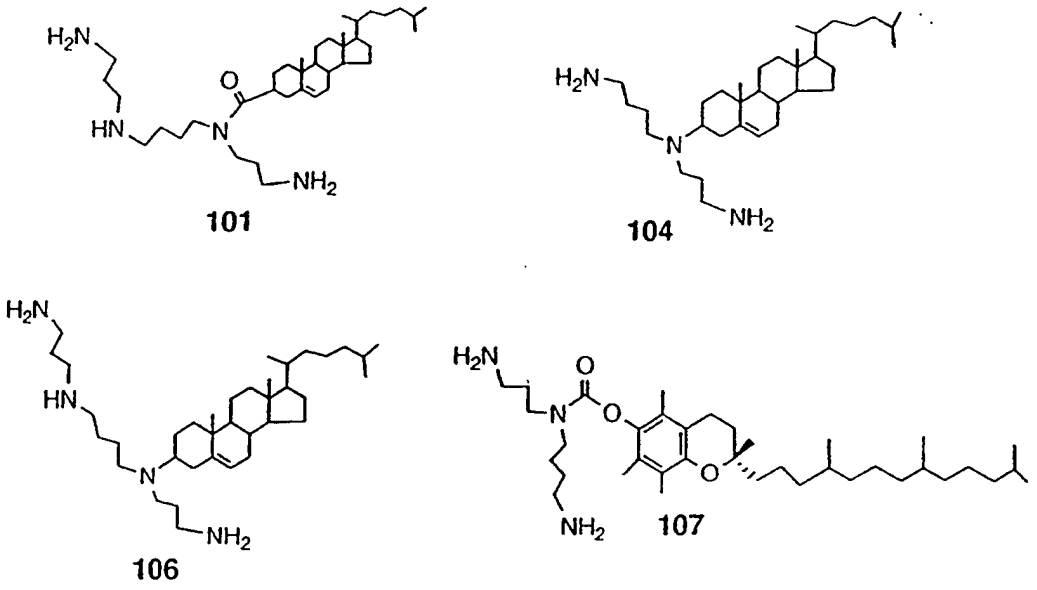


FIG. 1B

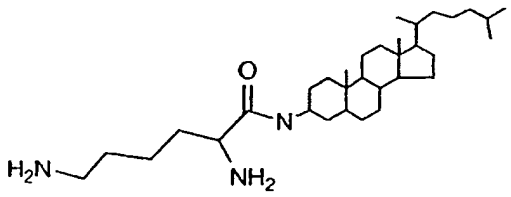
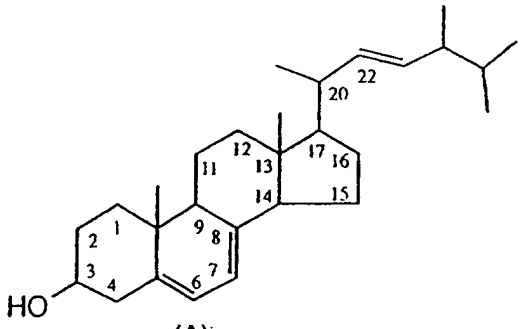
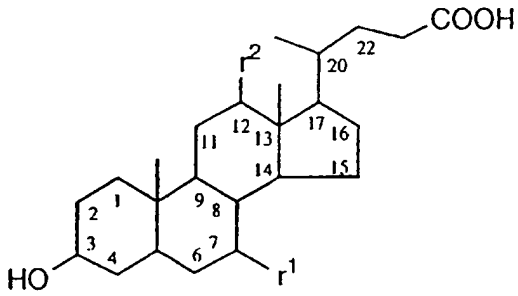


FIG. 1C



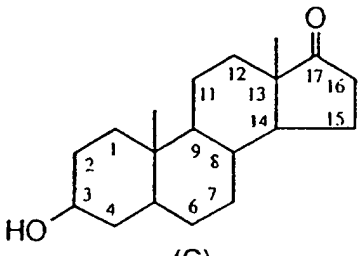
(A)

Ergosterol (double bonds as shown)
Ergosterol B1 (Δ 8, 9; Δ 14, 15; Δ 22, 23)
Ergosterol B1 (Δ 6, 7; Δ 8, 14; Δ 22, 23)
Ergosterol B1 (Δ 7, 8; Δ 14, 15; Δ 22, 23)
Lumisterol (9β-H isomer of ergosterol)



(B)

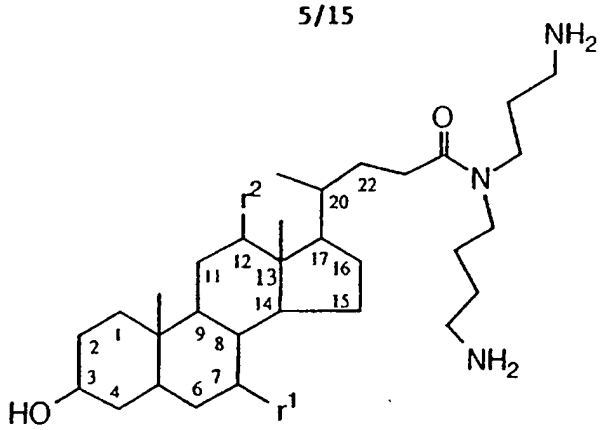
Cholic Acid r^1, r^2 = OH
Desoxycholic Acid r^1 = H, r^2 = OH
Chenodesoxycholic Acid r^1 = OH, r^2 = H
Lithocholic Acid r^1, r^2 = H



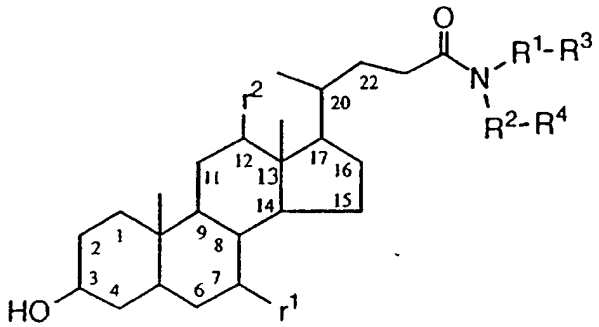
(C)

Androsterone

FIG. 2



(A)



(B)

Cholic Acid Derivative r^1, r^2 = OH
Desoxycholic Acid Derivative r^1 = H, r^2 = OH
Chenodesoxycholic Acid Derivative r^1 = OH, r^2 = H
Lithocholic Acid Derivative r^1, r^2 = H

FIG. 3

Transacylati n Reaction.

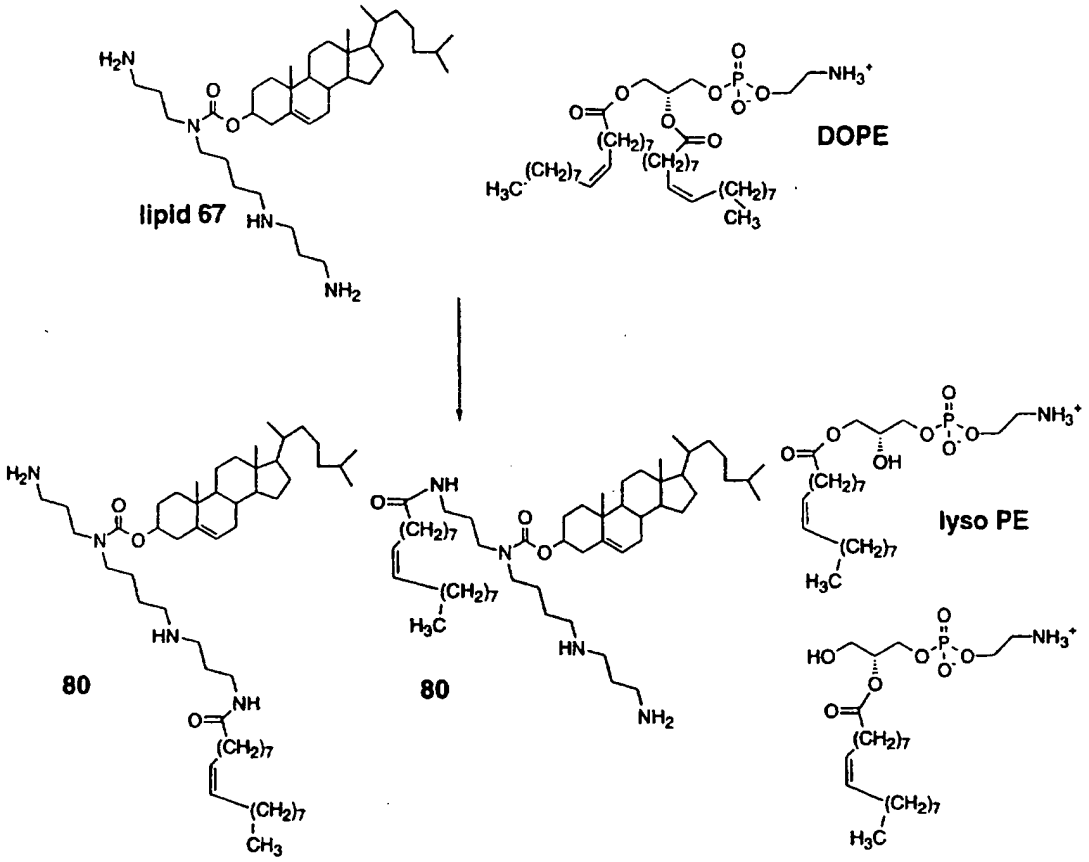
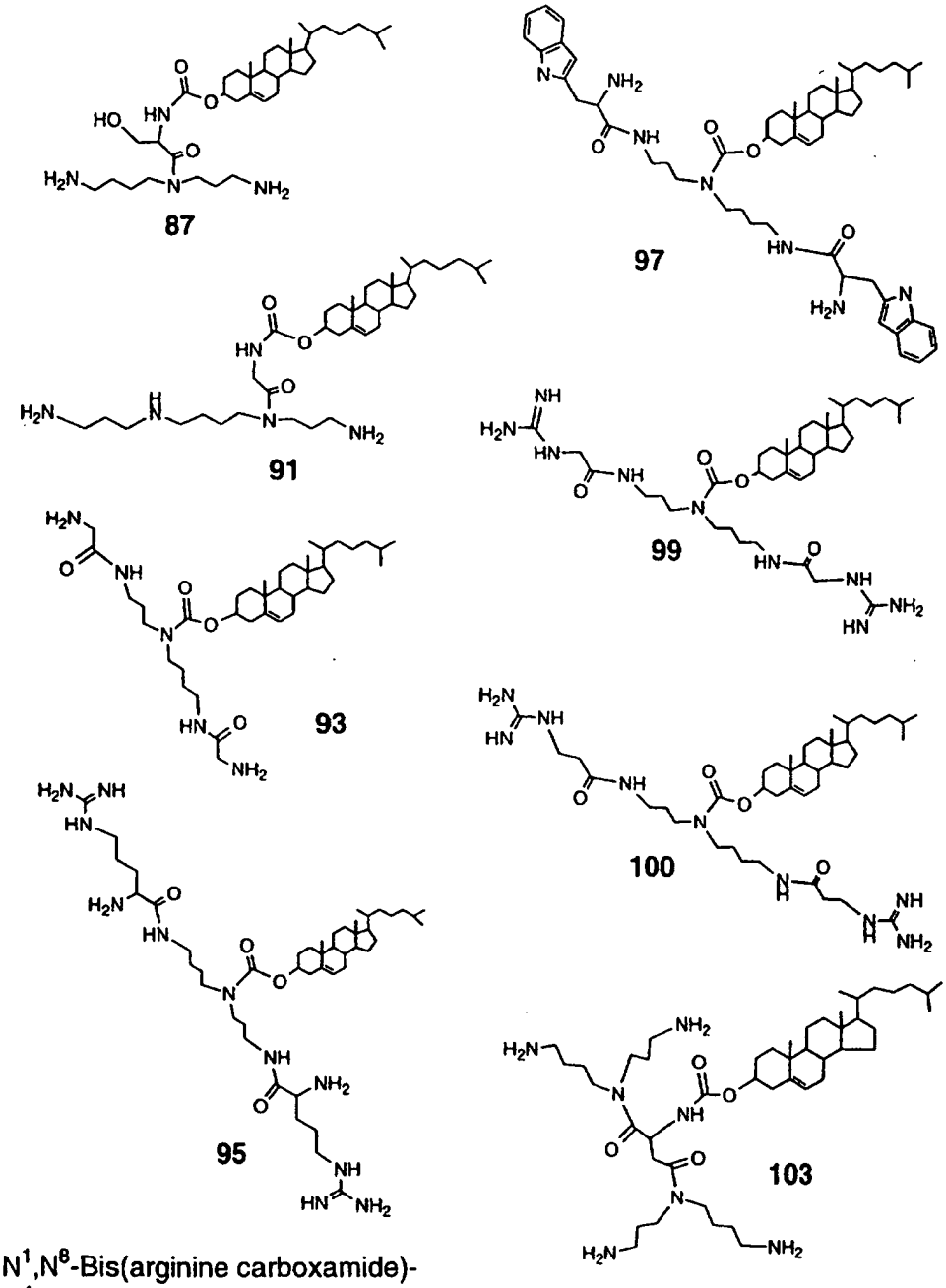
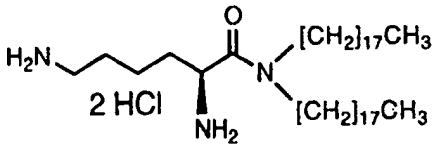


FIG. 4



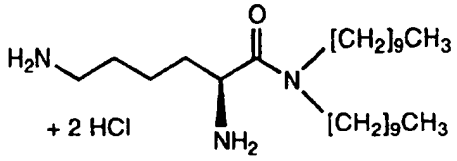
N^1,N^8-Bis(arginine carboxamide)-
N^4-spermidine cholesteryl carbamate

FIG. 5



43

N,N-dioctadecyllysineamide diHCl salt



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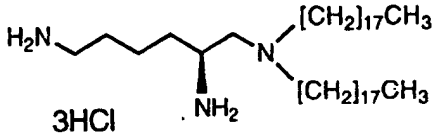
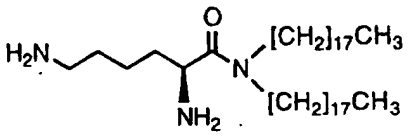


47

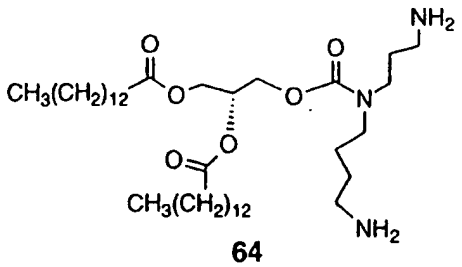
N^1,N^1-dioctadecyl-1,2,6-triaminohexane tri HCl salt



73

N,N-dioctadecyllysineamide

O



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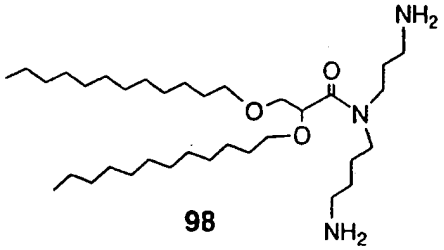

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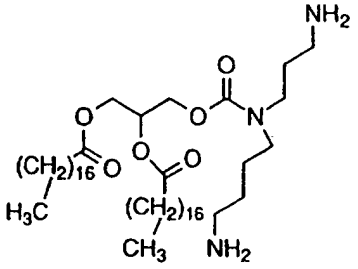

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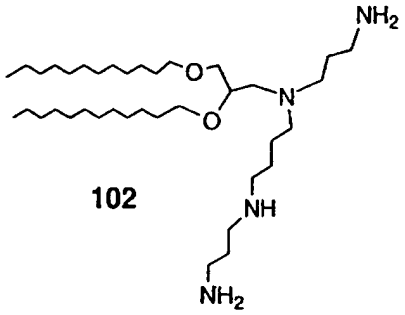
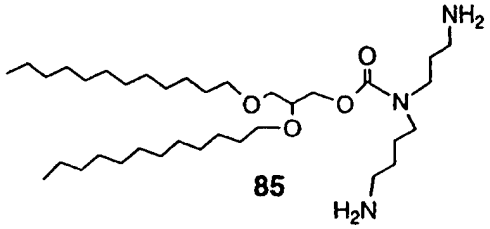


102

N^4-spermine-2,3-dilauryloxypropylamine



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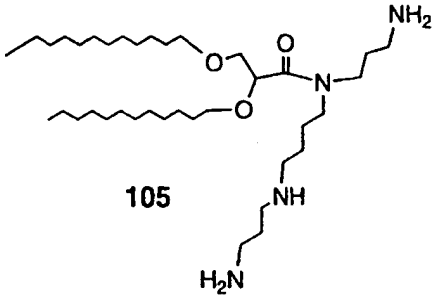

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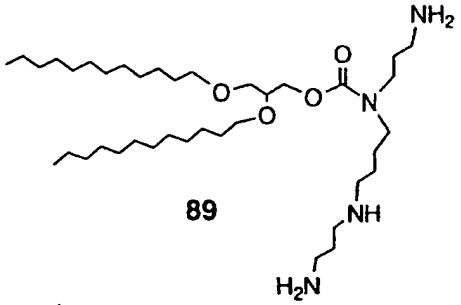
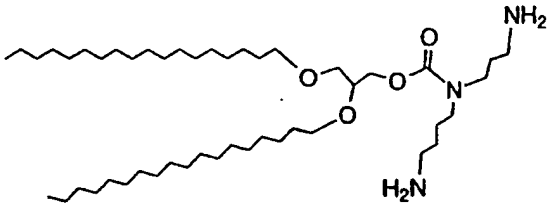


89

1-(N^4-spermine)-2,3-dilaurylglycerol carbamate



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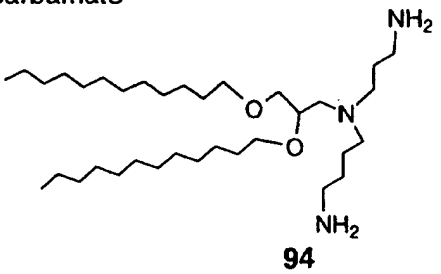
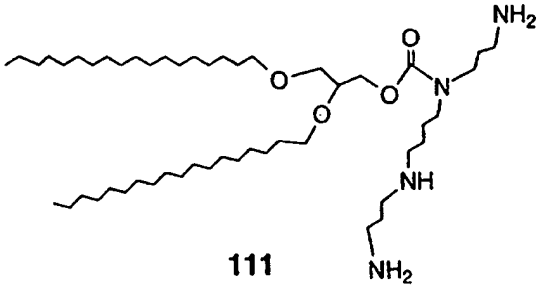


94

N^4-spermidine-2,3-dilauryloxypropylamine



111

FIG. 7

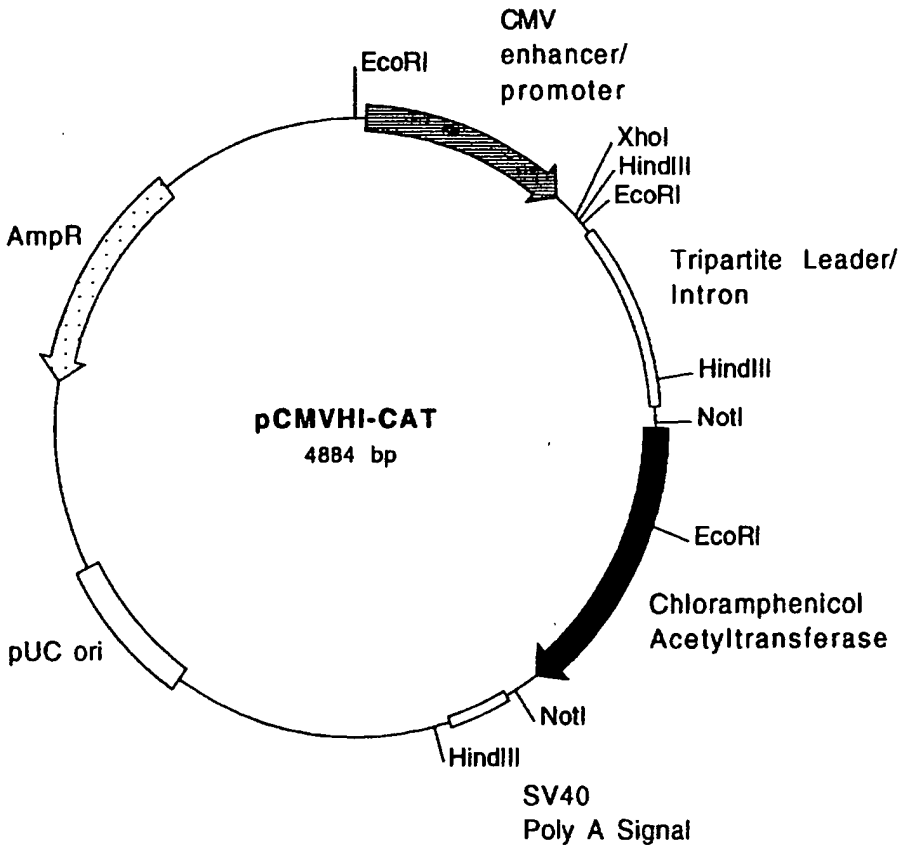

CMV
enhancer/
promoter
EcoRI
XhoI
HindIII
EcoRI
Tripartite Leader/
Intron
HindIII
NotI
EcoRI
Chloramphenicol
Acetyltransferase
NotI
HindIII
SV40
Poly A Signal
pUC ori
AmpR
pCMVHI-CAT
4884 bp

Fig. 8

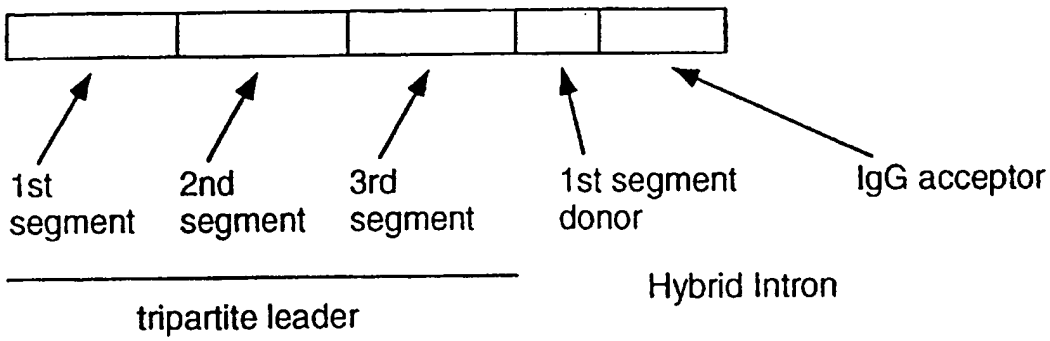

1st segment
2nd segment
3rd segment
1st segment donor
IgG acceptor
tripartite leader
Hybrid Intron

Fig. 9

Fig. 10A

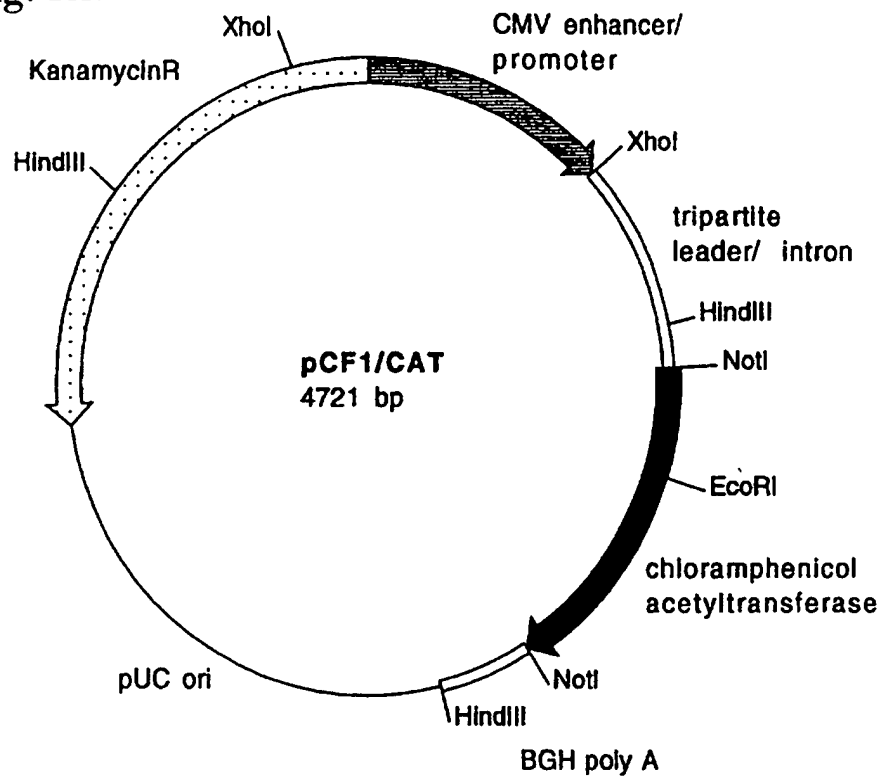
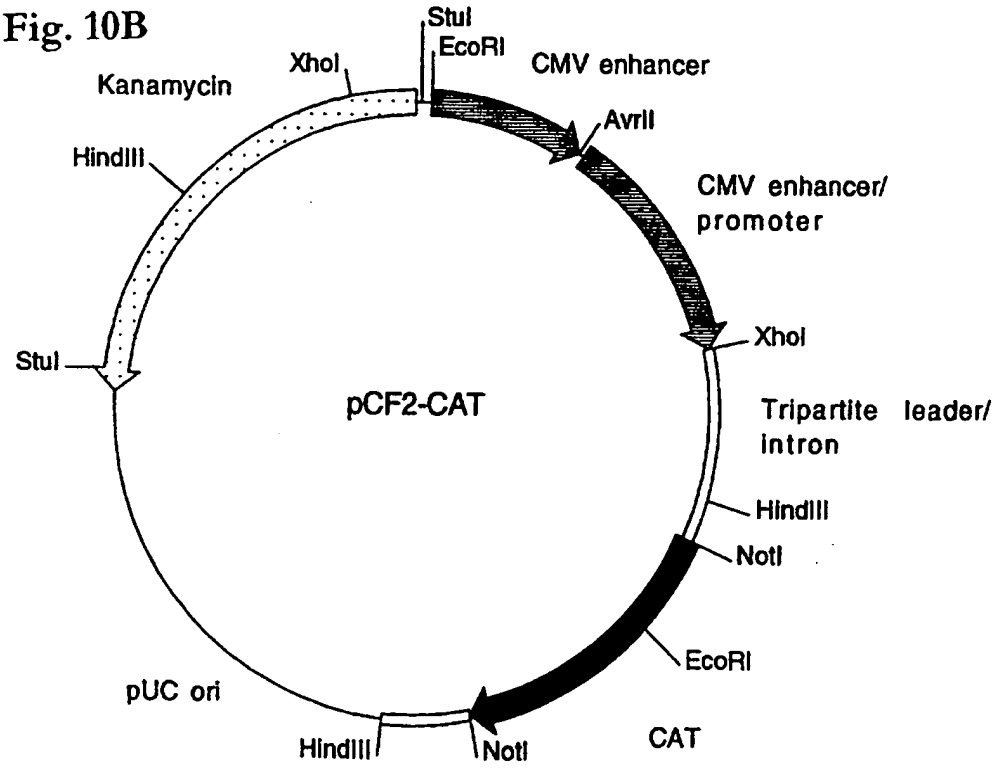


Fig. 10B



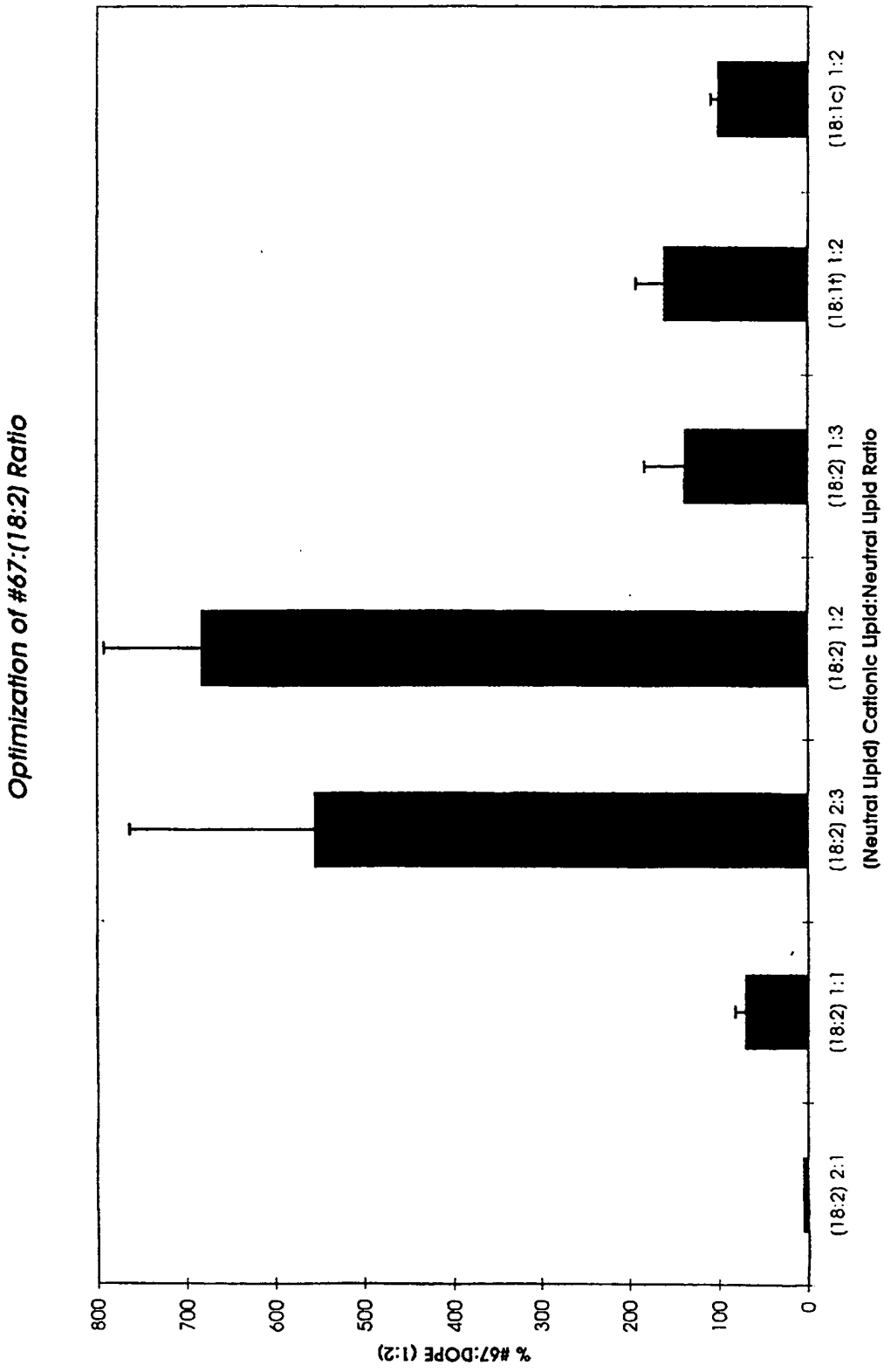

Optimization of #67:(18:2) Ratio
% #67:DOPE (1:2)
800
700
600
500
400
300
200
100
0
(18:2) 2:1
(18:2) 1:1
(18:2) 2:3
(18:2) 1:2
(18:2) 1:3
(18:1t) 1:2
(18:1c) 1:2
(Neutral Lipid) Cationic Lipid:Neutral Lipid Ratio

FIG. 11

Combinations of C18:3 and C16:0, C18:1 PE on Cos-1 Cells

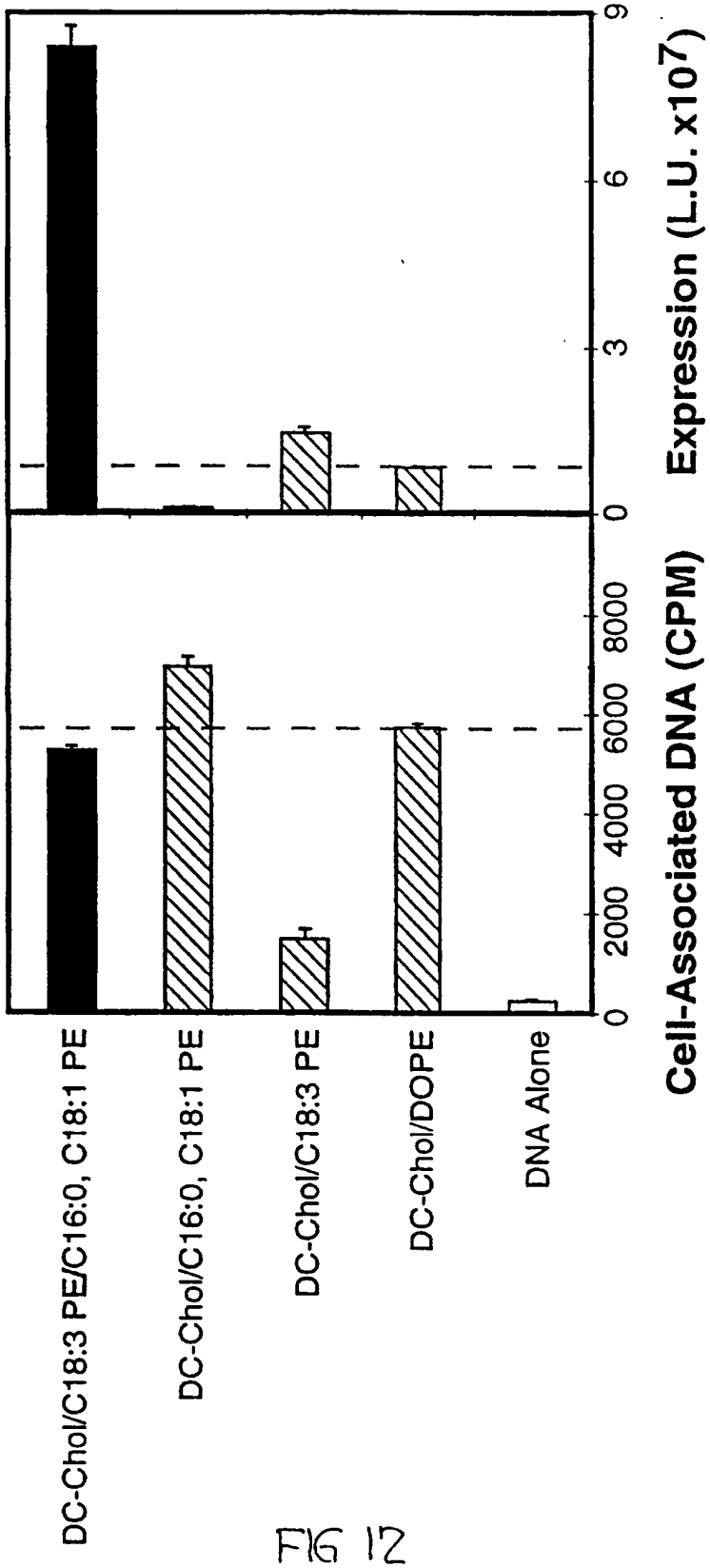


FIG 12

Expression Data for GL-67/PE Analogs *In Vivo*

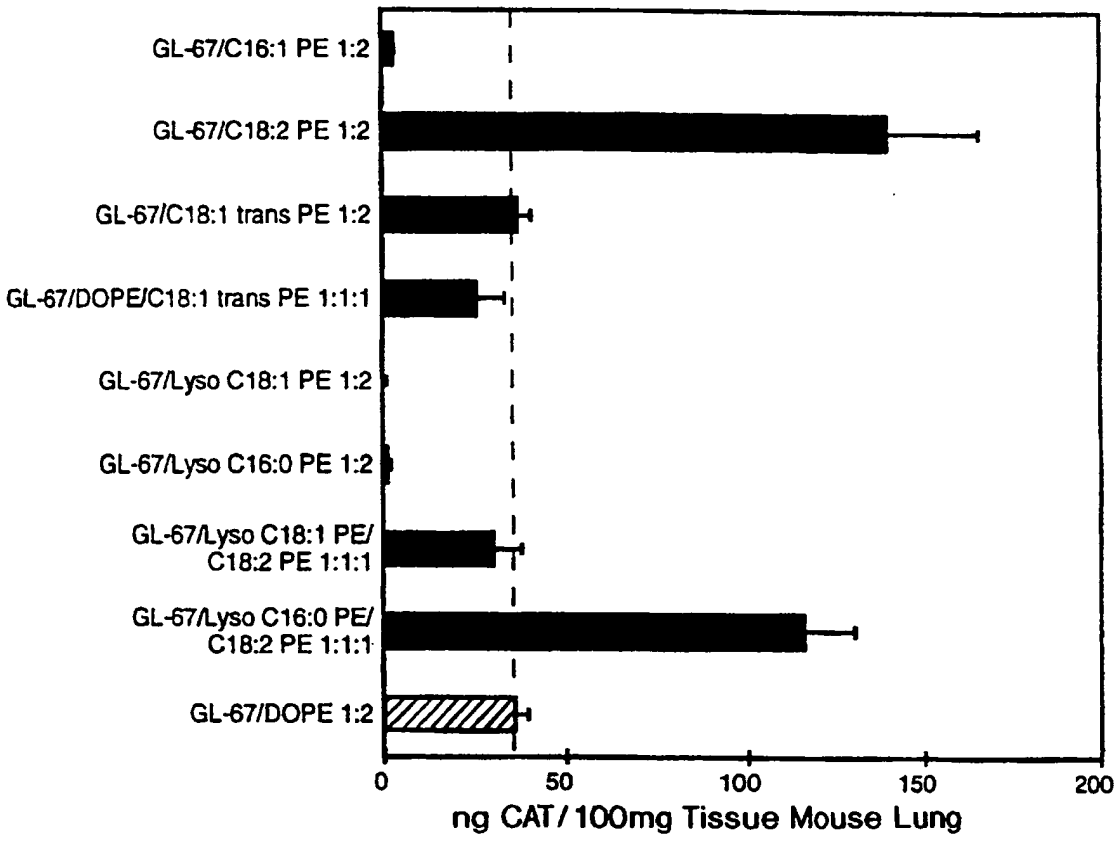


FIG. 13

INTERNATIONAL SEARCH REPORT

Intern ıal Application No
PCT/US 97/09142

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K9/127 C12N15/88

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X, L	WO 96 18372 A (GENZYME CORPORATION) 20 June 1996 see page 7, line 1 - page 9 see page 42, line 16 - page 43, line 7 see page 75 - page 80; example 3	5-7
P,Y	see claim 7 the priority claim of the present application might not be justified. ---	1-4,8-12
T	WO 97 29118 A (TRANSGENE S.A.) 14 August 1997 see claims 5-23 ---	1-12
Y	WO 91 15501 A (YALE UNIVERSITY) 17 October 1991 see claims 1,3 --- -/--	1-4,8-12

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 October 1997	31.10.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Benz, K

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INTERNATIONAL SEARCH REPORT

Intern nal Application No
PCT/US 97/09142

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	J. WANG ET AL. : "synthesis of multivalent cationic cholesteryl lipids for use as gene delivery vehicles" PROC. INT. SYMP. CONTROL. REL. BIOACT. MATER., vol. 22, 1995, pages 414-415, XP002017303 see the whole document	1-12
A	D. MORADPOUR ET AL.: "efficient gene tranfer into mammalian cells with cholesteryl-spermidine" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 221, no. 1, 1 April 1996, ORLANDO (US), pages 82-88, XP002031544 see the whole document see page 84; figure A	1-12
A	J.H. FELGNER ET AL.: "enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations" THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 4, 28 January 1994, BALTIMORE (US), pages 2550-2561, XP002010485 cited in the application see the whole document	1-12

1

INTERNATIONAL SEARCH REPORT

information on patent family members

Inten nal Application No
PCT/US 97/09142

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		AU 4516196 A	03-07-96
		EP 0799059 A	08-10-97
WO 9729118 A	14-08-97	FR 2744453 A	08-08-97
WO 9115501 A	17-10-91	US 5279833 A	18-01-94